

PRIMING AND MAINTENANCE OF *MYCOBACTERIUM TUBERCULOSIS* SPECIFIC T
CELL RESPONSES

by

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Vanja Lazarevic, Ph.D.

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1. ABSTRACT

Mycobacterium tuberculosis is the leading cause of death due to a single infectious agent worldwide. Immune response elicited against the bacterium is sufficient to control, but not to eliminate the pathogen leading to the establishment of asymptomatic state called latency. Currently very little is known about conditions that are required to prime robust, protective immune response in the initial stages of infection. We hypothesized that CD40 ligation is essential for the priming of strong Th1 response and for the subsequent control of *M. tuberculosis* infection. Furthermore, it is unknown how persistent exposure to mycobacterial antigens affects the functionality of T cells. Our initial thought was that the reason why immune response cannot eliminate *M. tuberculosis* and why reactivation occurs is due to T cell exhaustion as a result of continuous antigenic stimulation. Since the goal of every vaccine strategy is to elicit long-lasting and protective immunity, it is necessary to define the factors required for the development and maintenance of effector and memory T cell responses. We hypothesized that CD4 help and IL-15 are required for the development of functional CD8⁺ memory responses. Our results indicate that in murine model of tuberculosis CD40 ligation on

antigen presenting cells either by host- or mycobacterium-derived ligands, is essential for the induction of robust IFN- γ T cell response resulting in protection against disease and death. Once a strong immune response is elicited, long-term control of *M. tuberculosis* infection is mediated by dynamic changes in the frequency and types of T cell effector functions. We identified that IL-15 was not essential for the proliferation of CD4⁺ and CD8⁺ T cells or for the maintenance of their effector functions after primary and secondary *M. tuberculosis* infection. Our findings indicate that CD4 help was required during priming of effector CD8⁺ T cells and during secondary infection for the functional and durable memory CD8⁺ T cell responses. Collectively, the findings presented in this thesis broadened our understanding of what factors are essential for the generation and maintenance of functional effector and recall responses following *M. tuberculosis* infection.

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2. INTRODUCTION

2.1. Historical perspective

Mycobacterium tuberculosis has deep roots in the history of human civilization. DNA unique to *M. tuberculosis* complex has been detected in the lungs of an Egyptian mummy (1550 - 1080 BC) (1), and also in the body of a female mummy from Peru dating 1000 years ago (2). In Greek history, tuberculosis was described as “phthisis” (consumption), and it was noted in Hippocrates scripts (460 BC) that tuberculosis was well spread in Greek society, and that it was almost always fatal. For the longest time it was believed that “modern” *M. tuberculosis* emerged after humans adapted to the stationary mode of living and embraced agricultural practices as the means for survival (3). It was postulated that *M. bovis*, a bovine pathogen, “jumped” the species barrier and adapted to humans. One reason for such a belief was that the host range of *M. bovis* is very broad while *M. tuberculosis* is primarily a human pathogen (4). However, the most recent evolutionary study carried out by Brosch *et al.* revealed that the ancestor of today’s *M. tuberculosis* was specifically a human pathogen, and that *M. bovis* is relatively “new”, derived after successive loss of genetic material resulting in at least nine distinct deletions in the *M. tuberculosis* genome (4).

Well adapted to the living inside the human host, *M. tuberculosis* exploited changes in human society and used them to ensure successful spread amongst human population. It was not until 1882, that the causative agent of the “white plague” was identified as a bacillus by the name *Mycobacterium tuberculosis*. The exceptional microbiologist, Robert Koch, was responsible for this miraculous finding. He had isolated the causative agent of tuberculosis in humans and cattle, grew it in a pure culture, and reproduced the disease after inoculation of *M. tuberculosis* into

naïve animals. The Koch's postulates, as they are known today, are the basis of modern microbiology. In 1944, Waksman and colleagues opened the area of anti-tuberculosis chemotherapy after streptomycin, the first antibiotic effective against tuberculosis, was discovered (5). Very shortly, isoniazid (1952), rifampicin (1965), ethambutol (1968) and pyrazinamide (1970) were developed initiating a revolution in the treatment of tuberculosis (6). Prior to the advent of anti-mycobacterial therapy, tuberculosis had a 50% death rate.

Attempts to control tuberculosis by vaccination were initiated very soon after its identification by Robert Koch. However, it was the live, attenuated *M. bovis* vaccine by Albert Calmette and Camille Guérin that was shown to be effective in a series of animal models, and it was introduced as a human vaccine in 1921 (7). Such fast development in anti-tuberculosis treatment led Waksman to believe that “the ancient foe of man, known as consumption, the great white plague, tuberculosis, or by whatever other name, is on the way to being reduced to a minor ailment of man. The future appears bright indeed, and the complete eradication of the disease is in sight.” (5, 8). Yet, despite the global immunization with BCG vaccine and the existence of anti-tuberculosis antibiotics for the past 60 years, *M. tuberculosis* still remains the most dangerous foe of man responsible for infecting one third of the world's population and causing two million deaths each year, which translates to 4,931 deaths per day (9). The world is still very far from reaching its ultimate goal of tuberculosis eradication. In fact, the epidemic of tuberculosis in many countries prompted the World Health Organization to declare tuberculosis a global health emergency, and introduced directly observed therapy (DOTS) as an attempt to prevent the tuberculosis epidemic spiraling out of control.

The most intriguing question remains: “How is it that *M. tuberculosis* is not eliminated?” The reasons are multifactorial and we are partly to blame. Although anti-tuberculosis

chemotherapy is effective against *M. tuberculosis*, it takes six to nine months of treatment with double or triple antibiotic therapy to control active tuberculosis. Most patients are unwilling to complete such lengthy and complex regimens, and interrupt therapy once their condition is improved leading to relapse of the disease and emergence of multi-drug resistant TB. Socioeconomic and political changes that lead to the breakdown of health care and government systems, such as wars, refugees, poverty, malnutrition, and overcrowding will lead to the establishment and spread of *M. tuberculosis* epidemics. However, the most important factor in the tuberculosis eradication is the ability of *M. tuberculosis* to persist and survive in the adverse environment of its host and avoid elimination by host's immune system and harsh antibiotic treatments. This persistence results in an enormous reservoir of infected but asymptomatic persons, all with a risk of developing active tuberculosis. Such a reservoir makes it almost impossible to eradicate tuberculosis. The next section describes the genetic adaptation of and mechanisms by which *M. tuberculosis* resists and prevents its eradication by the host.

2.2. Survival strategies of *Mycobacterium tuberculosis*

2.2.1. The initial response

M. tuberculosis infection is acquired through inhalation of aerosolized droplets containing the bacilli generated by a person with active tuberculosis. Droplets 1 – 5 µm in diameter avoid mechanical elimination by tracheal cilia, and settle in the alveolar spaces of the lungs, where they encounter alveolar macrophages. Using complement receptors CR1, CR3, CR4, mannose receptor, scavenger receptors, CD14 or surfactant Sp-A receptors, *M. tuberculosis* establishes successful infection of alveolar macrophages (MΦ) (10, 11). Current evidence

supports that the entry pathways of *M. tuberculosis* do not have a major survival advantage or a huge impact on the intracellular growth of mycobacteria (10). Such promiscuous use of receptors indicates that *M. tuberculosis* is determined to enter the intracellular environment of the macrophages, and hide from the potent immune response that is about to be generated.

Inside resting macrophages, *M. tuberculosis* interferes with the regulators of intracellular trafficking and imposes phagosomal maturation arrest by multiple mechanisms. Normally, a phagosome formed around inert latex beads and many bacteria undergoes progressive acidification (pH 4.5) and fusion with lysosomes containing potent hydrolyases leading to the destruction of ingested material. However, D'Arcy Hart and colleagues were first to note that *M. tuberculosis* successfully interferes with this process, and while maintaining an active and dynamic relationship with endocytic compartment, it blocks the fusion and interaction with pre-existing lysosomes (12). The block occurs between the maturation stages controlled by small GTP-binding proteins Rab5 (early endocytic) and Rab7 (late endosomal) (13). *M. tuberculosis* possesses many bioactive lipids, and lipoarabinomannan (LAM) has been implicated in the phagosomal maturation arrest (14). It is believed that LAM inhibits Ca^{2+} /calmodulin- and Rab5-mediated recruitment of phosphatidylinositol-3-kinase (hVPS34) to the mycobacteria-containing phagosomes (reviewed in (13)). This hVPS34 kinase produces phosphatidylinositol-3-phosphate which is essential for the binding of early endosomal autoantigen 1 (EEA1) and tethering it to the phagosomal membrane (15-17). EEA1 together with trans-Golgi network SNARE Syntaxin 6 then delivers V_0H^+ ATPase to the phagosomal membrane leading to the progressive acidification of the phagosome and activation of the delivered lysosomal hydrolases (13). Thus, *M. tuberculosis* derived LAM blocks the vesicle transport between trans-Golgi network and phagosomes by interfering with phosphatidylinositol-3-phosphate pathway (13). However,

survival of *M. tuberculosis* inside the macrophages is dependent on maintaining the active communication with early endosomal pathway by which it acquires essential nutrients, particularly iron. Another *M. tuberculosis* derived lipid phosphatidylinositol mannoside (PIM) is characterized as having fusion-promoting properties. It was found that PIM specifically stimulates fusion of early endosomes with mycobacteria-containing phagosomes in an ATP, cytosol and Rab dependent manner (18).

Another mechanism by which *M. tuberculosis* can interfere with phagosomal maturation is through interaction with TACO (tryptophan aspartate rich coat protein). TACO shares approximately 35% homology to coronin protein derived from *Dictyostelium* slime mold (19). Genetic studies with *Dictyostelium* suggested that coronin may be implicated in phagocytosis and particle uptake by this organism (20). Based on the homology results between TACO and coronin, it was postulated that phagocytosis may trigger TACO acquisition and retention on mycobacteria containing phagosomes. Although not proven experimentally, it was proposed that TACO may be functioning to prevent the fusion of lysosomes with the plasma membrane, and thus retention of TACO on the mycobacteria-containing phagosomes identifies this membrane non-fusogenic with lysosomes (19).

M. tuberculosis also interferes with signal transduction pathways of the host cells by encoding several serine-threonine kinases, such as protein kinase G, which modulate the intracellular membrane trafficking and prevent lysosomal delivery to the phagosomes harboring viable mycobacteria (21).

The net result of these inhibitory mechanisms is uncontrolled replication of *M. tuberculosis* inside the host cells, which is seen in the first two to three weeks of infection until cell mediated immunity is induced.

2.2.2. Induction of the immune response and subsequent adaptation to the hostile environment of activated macrophages

In addition to invading resident alveolar macrophages, *M. tuberculosis* also infects dendritic cells (DC) that are patrolling the lung parenchyma in search of invading pathogens (Figure 1). Infected DC will mature, leave the lung, and migrate to the regional lymph nodes where they prime naïve T cells. Activated T cells travel to the lung where they recognize infected macrophages. Host immune response against *M. tuberculosis* is initiated and results in the formation of organized structures called granulomas (Figure 1). The main function of the granuloma is to seal off the focus of infection by enclosing the infected macrophages and to enable intimate T cell – macrophage contacts. *M. tuberculosis*-specific T cells deliver potent cytokines such as IFN- γ and TNF, which synergistically activate infected macrophages to elaborate toxic reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). Suddenly, *M. tuberculosis* has to deal with multiple adverse effects that are imposed upon it by activation of macrophages and granuloma formation.

2.2.3. Life within activated macrophages

IFN- γ and TNF- α will overcome mycobacterial phagosomal maturation arrest mechanisms leading to the phagosome-lysosome fusion. *M. tuberculosis* counteracts by effectively producing ureases, which through generation of ammonia raise the phagolysosomal pH, and prevent the activation of lysosomal hydrolases (22). Gene expression microarray analysis captured the transcriptional responses of *M. tuberculosis* in macrophages from wild-type and NOS2-/- mice before and after immunological activation.

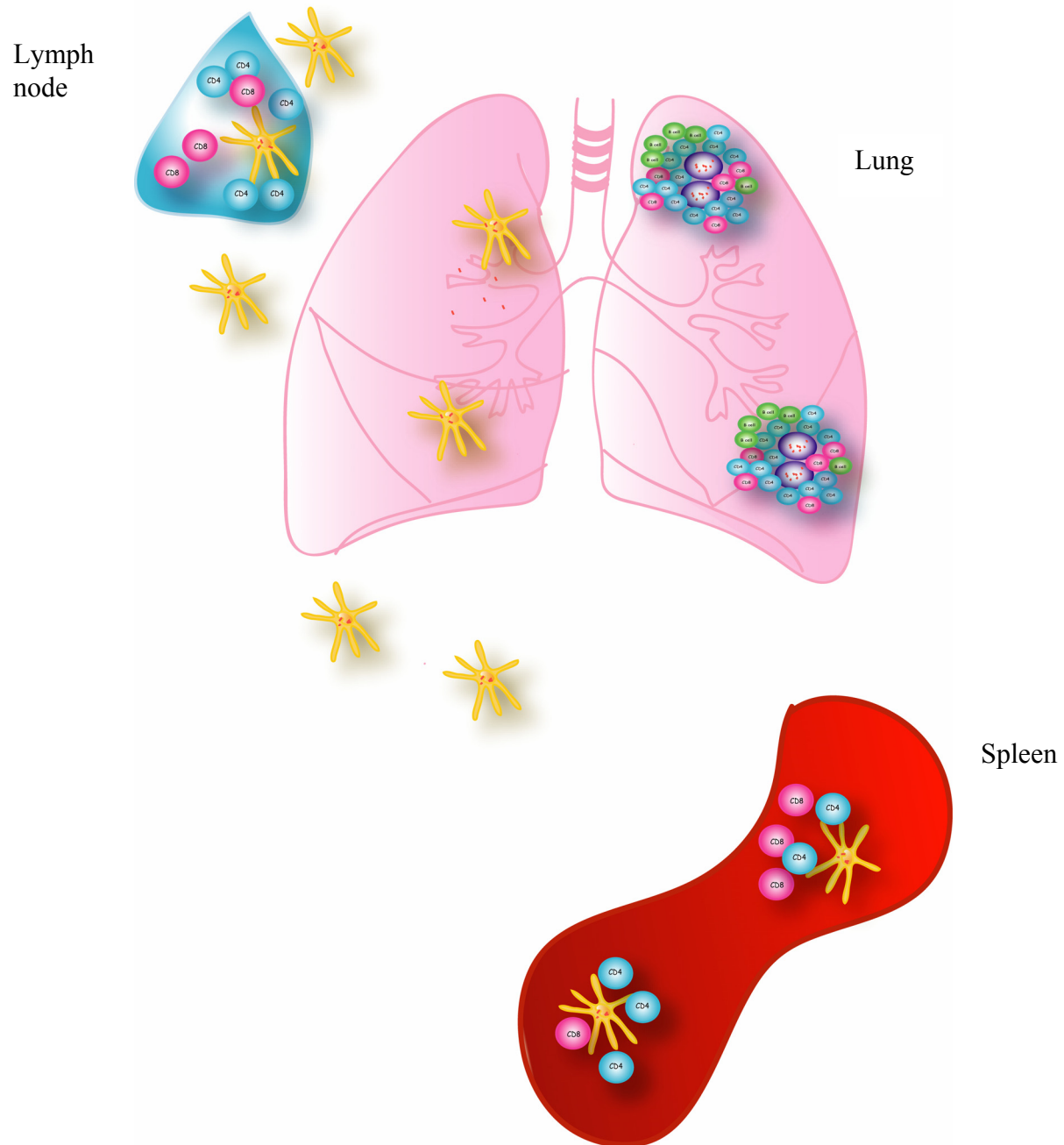


Figure 1 Initial response to *M. tuberculosis* infection

M. tuberculosis (red rods) infection is acquired through inhalation of aerosolized droplets containing tubercle bacilli. Once inside the lung, *M. tuberculosis* is engulfed by resident alveolar dendritic cells (yellow cells), which migrate to the regional lymph nodes and spleen to prime naïve T cells (CD4⁺ blue cells; CD8⁺ red cells). Activated T cells travel to the site of infection where they recognize infected macrophages (purple cells), and elaborate their effector functions, which will lead to the better control of *M. tuberculosis* infection. The net result is formation of granulomas, which allow intimate contacts between macrophages and T cells, and prevent further spread of infection.

Induction of enzymes involved in the β -oxidation of fatty acids indicates that *M. tuberculosis* relies on fatty acids as carbon and energy source. In addition, induction of sodium dodecyl sulfate (SDS)-regulated genes, and genes involved in the mycolic acid modification point to damage and repair of the cell wall (23, 24). Collectively, these results suggest that the phagosomal environment in which *M. tuberculosis* resides after immunological activation of macrophages is nitrosative, oxidative, hypoxic, nutrient-deficient and damaging to the *M. tuberculosis* cell wall (23, 24).

M. tuberculosis, however, encodes several enzymes that specifically serve as a defense against nitrosative and oxidative stress. In search for transposon mutants with hypersusceptibility to acidified nitrite, Darwin *et al.* identified five mutants that had insertions in proteasome-associated genes (25). Chemical inhibition of *M. tuberculosis* encoded proteasomes rendered bacteria susceptible to reactive nitrogen intermediates replicating the phenotype of proteasome mutants. Thus, mycobacterial-encoded proteasomes function to protect the organism against nitrosative stress. The mechanism of protection probably involves the degradation of irreversibly oxidized and nitrated proteins (25). In addition, *M. tuberculosis* *ahpC* gene encodes the peroxiredoxin alkyl hydroperoxide reductase subunit C (AhpC) which is capable of catabolizing peroxynitrate anion (ONOO^-), a potent oxidant formed between NO and O_2^- (26). Although the role of oxidative burst in the control of *M. tuberculosis* infection is debatable (see below), genetic analysis of *katG* *M. tuberculosis* mutants revealed that *M. tuberculosis* is equipped with ROS detoxification mechanisms, which contribute to its virulence. Although markedly attenuated in wild-type and NOS2^{-/-} mice, the *katG* deletion mutant was equally capable of replicating and persisting just like virulent *M. tuberculosis* inside the gp91^{phox}^{-/-} mice, which lack the gp91 subunit of NADPH oxidase (26). LAM, mycobacterial sulfatides and

phosphoglycolipid-1 (PGL-1) have all been shown to act as oxygen radical scavengers thus reducing the damaging effects of ROS (27, 28).

Since recognition of *M. tuberculosis*-infected macrophages has such a detrimental effect on mycobacterial survival inside activated cells, *M. tuberculosis* employs a whole spectrum of mechanisms by which it interferes with antigen processing and presentation by their host cells. It has been shown in several studies that *M. tuberculosis* components decrease MHC Class II mRNA synthesis by downregulating expression of Class II transactivator (CIITA), sequester immature MHC Class II heterodimers, and interfere with the normal trafficking of MHC Class II molecules by alkalization of MHC Class II compartment due to the increased production of mycobacterium encoded urease (29-33). In addition, *M. tuberculosis* inhibits macrophage responses to IFN- γ signaling in a Toll-like receptor (TLR) – 2 and myeloid differentiation factor 88 (MyD88)-dependent and independent fashion (34). Inhibition of IFN- γ signaling may result in diminished ability of macrophages to kill intracellular mycobacteria after stimulation with IFN- γ . *M. tuberculosis*-infected macrophages secrete IL-6, which render the surrounding, bystander, uninfected macrophages refractory to activation by IFN- γ suggesting that *M. tuberculosis* exploits the inhibitory property of IL-6 to evade eradication by robust immune response (35).

2.2.4. Life within the granuloma

As the immune response against *M. tuberculosis* becomes vigorous, the granuloma progressively matures, and it is thought to have stratified structure. The center of a granuloma is composed of a cheese-like, semi-solid material that is low in oxygen, and rich in lipids derived from dead host cells and bacteria (caseum). It is believed that inside this necrotic, caseous center, mycobacteria exist extracellularly (36). The center of the granuloma is surrounded by a layer of

partially activated or fully activated macrophages, which are enclosed by non-activated macrophages and T and B lymphocytes. The granuloma is mantled with fibroblasts which give the strength and support to the whole structure (36). Containment of *M. tuberculosis* within granuloma may lead to eventual calcification of the surrounding tissues, and within these encased structures mycobacteria can persist for decades, even for the life time of an individual without reactivating.

Assessment of *M. tuberculosis* within human lesions is limited to necropsy specimens. In a study by Opie and Aronson lesions from cadavers often contained acid-fast bacilli (37). The findings from their study demonstrated that homogenates from fibrocaseous lesions of the apex of the lung typically caused tuberculosis when injected into guinea pigs. In contrast, homogenates from calcified lesions seldom contained viable bacteria and were unable to cause tuberculosis in susceptible animals. Interestingly, they recovered viable, infectious mycobacteria from the apparently healthy looking lung tissues in half of the cases, suggesting that latent *M. tuberculosis* could exist outside granulomatous lesions (37). Many years later, *in situ* PCR analysis demonstrated the presence of *M. tuberculosis* genomic DNA in lung endothelial cells and type II pneumocytes indicating that *M. tuberculosis* can infect other cell types in addition to macrophages (38).

Surgical resection of TB lesions after chemotherapy revealed large number of acid fast bacilli observed by microscopy even months after patients on chemotherapy became sputum negative (39-41). Most lesions failed to yield cultivable bacilli, which led to hypothesis that these mycobacteria were “viable but not cultivable” or “not dead, but dormant” as a result of presence of inhibitors of mycobacterial growth in the tissues of antibiotic treated patients (42). Indeed, after careful and extensive washing of resected lung tissue, the rate of successfully recovered

mycobacteria increased (40). Mitchinson was first to hypothesize that the persistence of bacilli in the face of chemotherapy was attributed to physiological heterogeneity of bacteria in the lung tissues (5). This theory predicts that anti-TB drugs target and eliminate fast growing bacteria first, while the drugs are ineffective against intermediate and slow growing mycobacteria (5). Existence of bacteria with different susceptibilities to antibiotics could explain the need for the long term (at least 6 months) treatment with antibiotics to which patients must be subjected before infection is fully contained (5). Clinical evidence in support of Mitchinson's hypothesis came from a study in which pulmonary lesions defined as being "open and active" yielded drug-resistant bacteria cultured within 2 months, while bacilli from lesions that were classified as "dormant and closed" were cultivable only after prolonged incubation of 10 months and they were unvaryingly drug susceptible (43). Growing evidence indicates that the physiological and metabolic state of mycobacteria grown *in vitro* does not represent their state inside the lungs.

2.2.5. Modeling *M. tuberculosis* persistence under *in vitro* culture conditions

In Wayne model of dormancy, unshaken *M. tuberculosis* cultures were allowed to settle slowly through a self-generated oxygen gradient into the anaerobic conditions present at the bottom of the culture flask. As bacteria reached anaerobic environment, they underwent stage-specific cell cycle arrest and entered the state of non-replicating persistence (NRP) (44). Among strategies that appear to facilitate hypoxic NRP in *M. tuberculosis* are restriction of biosynthetic activity (almost total shut down of DNA and protein synthesis, while RNA synthesis is minimal), induction of enzyme systems that utilize alternative energy sources and development of mechanisms for stabilizing the bacterial cells and their contents (44). Dormant state induced by

oxygen deprivation is also characterized by loss of antibiotic sensitivity to isoniazid and rifampicin, and increased sensitivity to metronidazole (44).

One strategy to circumvent oxygen deficiency is to use alternative mechanisms for energy generation. RNA analysis on mycobacteria from anaerobic cultures revealed elevated levels of two enzyme systems, one leads to reductive amination of glyoxylate (isocitrate lyase) and the second one to nitrate reduction (nitrate reductase) (44). In addition, two more processes may play a protective role: induction of alpha-crystallin and thickening of cell wall. Alpha crystallin is expressed six hours after induction of hypoxia, and the major role of this protein with chaperone-like activity is to protect and reduce the rate at which essential proteins critical for survival need to be replaced (44). The whole process is orchestrated by a set of thirteen potential sigma factors whose expression is increased upon entry into hypoxic or stationary phase (44).

2.2.6. Modeling *M. tuberculosis* persistence *in vivo*

Most observations made using the Wayne model of persistence were replicated in the two mouse models of persistence, namely the low-dose and Cornell models of persistence.

In the low-dose model, mice are infected with a low dose of *M. tuberculosis* via aerosol route (~ 10 – 50 CFU), reviewed in (45). During the first three weeks of infection, mycobacteria replicate uncontrolled in the lungs of infected mice until induction of robust immune response. Infiltration of immune cells and elaboration of their effector mechanisms hinder bacterial replication, and bacterial numbers are stably maintained at 10^4 to 10^6 CFU per lung for the life of a mouse without any overt signs of disease. Spontaneous reactivation is rare, and experimental manipulations such as neutralization of TNF, inhibition of NOS2 or depletion of CD4⁺ T cells

are essential for reactivation to occur leading to death of mice (46-49). The only caveat with this model is that the bacterial load at which latency is established is significantly higher than in humans leading to the progressive immunopathology and deterioration of lung architecture (45).

In the Cornell model of persistence, mice are infected with *M. tuberculosis* and latency is established by administration of isoniazid and pyrazinamide for twelve weeks (50). Such treatment will reduce, but not eliminate *M. tuberculosis*. Following completion of antibiotic therapy no viable bacteria were cultured after plating multiple organ homogenates, and it was impossible to transfer infection to susceptible guinea pigs after injection of the organ extracts from antibiotic treated mice (50). Hence, latency is established as the presence of infection cannot be demonstrated by any available methods. However, after several months, a significant proportion of antibiotic treated mice spontaneously reactivated, and with immunosuppressive corticosteroid treatment rate of reactivation increased to 100% (50). The main caveat of the Cornell model is that the success of reactivation is not always reproducible amongst different laboratories. Factors such as time of initiation of antibiotic treatment, types of antibiotics used, and duration of treatment can influence the rate of reactivation (47).

Although the mouse granuloma performs the same function of preventing the spread of infection, it is important to note that the mouse granuloma is structurally different from the human granuloma. It lacks the necrotic, caseous center in which mycobacteria are believed to reside during latent infection in humans. Some critics of the mouse model of human latency believe that conditions inside the mouse granuloma do not accurately represent the insults to which mycobacteria are exposed inside human granuloma, and development of non-human primate models of tuberculosis will definitely lead to the better understanding of human latency. Nevertheless, generation of a whole library of *M. tuberculosis* mutants and microarray analysis

of the mouse lung tissues following *M. tuberculosis* infection yielded important insights into the requirements of *M. tuberculosis* for existence and persistence during latent infection.

Studies of *M. tuberculosis* mutants in the mouse models of persistence led to the classification of attenuated mutants into several phenotypic categories: severe growth in vivo mutants (*sgiv*), growth in vivo mutants (*giv*), persistence (*per*), pathology (*pat*) and dissemination (*dis*) mutants, reviewed in (9).

2.2.6.1. Phenotypic characterization of “severe growth in vivo” *M. tuberculosis* mutants

Most of *sgiv* mutants have severe growth defects in nutrient-poor media, murine or human macrophages and they are unable to establish infection in the lungs of infected mice. The severe growth defect of *sgiv* stems from bacterial inability to obtain essential nutrients necessary for the survival and replication inside phagosomal environment (9). Magnesium acquisition appears to be essential for *M. tuberculosis* virulence, hence a *mgt* (magnesium transporter) mutant was unable to replicate under low magnesium and slightly acidic conditions. Consequently, *mgt* mutant could not survive inside macrophages and showed severe growth defect in the lungs and spleens of infected mice (51). Many auxotrophic mutants of *M. tuberculosis* with deletions in leucine (*leuD*), lysine (*lysA*) proline (*proC*) and tryptophan (*trpD*) biosynthetic pathways belong to this category of mutants (52-55).

2.2.6.2. Phenotypic characterization of “growth *in vivo*” *M. tuberculosis* mutants

These mutants are characterized by limited replication inside the lungs; they have attenuated phenotype leading to the increased survival of infected mice (9). Some *giv* mutants are auxotrophic mutants; i.e. they show impairment in one of the important biosynthetic pathways that are necessary to ensure survival of *M. tuberculosis* under nutrient and oxygen deficient conditions. One such mutant is characterized by the absence of glutamine synthetase gene (*glnA*), which is necessary for nitrogen metabolism during persistence under anaerobic conditions (56). Since acquisition of iron is very important for intracellular survival of mycobacteria, deletion in one of the bacterial mycobactins (*mbtB*) led to decreased growth under iron-deficient conditions both *in vitro* and within macrophages. Hence, it was not surprising when *MbtB* mutant showed severe attenuation in its virulence and significantly limited replication in the lungs of infected mice (57). It was unexpected to discover the importance of vitamin B5 *de novo* biosynthesis in the virulence of *M. tuberculosis*. Deletion of *panC* and *panD* mutants rendered mycobacteria sensitive to the adverse insults in infected lungs, and *panCD* mutants were highly attenuated in mouse survival studies (58). Mycobacterial tyrosine kinases and phosphatase act as virulence factors by interfering with host cell signaling. A *mptpB* mutant strain of *M. tuberculosis* had impaired ability to survive in activated macrophages and guinea pigs, but not in resting macrophages suggesting that mycobacterial phosphatase MptpB plays an important role in host-pathogen interaction (59). Two component regulatory proteins function in bacteria to sense and respond to changes in the extracellular environment. PhoP/PhoQ two component regulatory proteins are necessary for *M. tuberculosis* survival *in vivo* as a *phoP* deletion mutant had reduced growth in the liver, lung and spleen of infected mice, which is

characteristic of *giv* mutants (60). As most bacterial pathogens export their virulence factors through the use of secretion systems, it was postulated that *secA* mutant of *M. tuberculosis* will show attenuated phenotype *in vivo*. Indeed, *secA* mutants were more sensitive to macrophage effector mechanisms as superoxide dismutase (SOD) could not protect mycobacteria from reactive oxygen species as secretion of SOD is SecA-dependent (60). Camacho *et al.* identified 16 *giv* mutants some of which were defective in the synthesis of the complex cell wall-associated lipids such as phthiocerol and phenolphthiocerol derivatives (61). Such mutants exhibited profound morphological changes and alterations in the cell wall, which may have made the mutants more sensitive to the reactive oxygen and nitrogen intermediates (61).

2.2.6.3. Phenotypic characterization of “persistence” *M. tuberculosis* mutants

Persistence mutants grow well during initial stages of infection; however, these mutants fail to resist the onslaught of the immune response, and cannot persist once cell mediated immunity is initiated (9). These mutants have revealed virulence factors that enable *M. tuberculosis* to withstand host adaptive immune responses. Genes involved in “cording” (*pcaA*) or encoding isocitrate lyase (*icl*), phospholipases (*plcABCD*), and *dnaE2* were all shown to have reduced growth after the onset of acquired immunity (9). “Cording”, i.e. formation of microscopic ropes or bundles by mycobacteria has always been associated with the most virulent phenotypes. A novel mycolic acid cyclopropane synthetase encoded by *pcaA* gene was shown to be required for cording, persistence and virulence of *M. tuberculosis* during latent infection (62). These findings underscore the importance of cyclopropanated lipids in mycobacterial pathogenesis. Isocitrate lyase (*icl*) mutant is one of the best characterized persistence mutants. This enzyme is known to be essential for the metabolism of fatty acids, and reduced survival of

icl mutants after initiation of immune response indicates that long term survival of *M. tuberculosis* inside the infected host relies heavily on fatty acid metabolism (63). The strongest link between the requirement for isocitrate lyase and the immune status of the host was established by the restored virulence of *icl* mutant in IFN- γ knockout mice (63). When microorganisms encounter a nutrient limited environment they slow their growth dramatically and reduce the level of RNA and protein synthesis. In *M. tuberculosis* this starvation program is mediated by hyperphosphorylated guanine nucleotides, or pppGpp, which are synthesized and hydrolyzed by Rel_{Mtb} protein (9). *M. tuberculosis* mutants that lack this protein are defective in long-term survival both *in vitro* and *in vivo* (64). Hence, elimination of the major bacterial mechanism for starvation adaptation leads to the perishing of mycobacteria inside the hosts. Another mechanism by *M. tuberculosis* resists eradication by the immune response is possession of an efficient error-prone DNA repair system (65). Genetic studies have shown that drug resistance emerged more frequently in wild-type and complemented strains of *M. tuberculosis* than in *dnaE2* mutant suggesting that the action of this polymerase may contribute the emergence of drug resistance *in vivo* (65).

2.2.6.4. Phenotypic characterization of “pathology” *M. tuberculosis* mutants

Pathology mutants do not show growth defects after initiation of immune response; instead it is believed these mutants elicit reduced immunopathology when compared to wild-type *M. tuberculosis* (9). The only mutants that showed reduced pathology and attenuated virulence were bacteria that lack one of regulatory factors such as SigH, SigE, SigA (66-68). It was reported that infection of mice with these mutants results in reduced infiltration of cytokine secreting T cells into the lungs and formation of fewer granuloma.

2.2.6.5. Phenotypic characterization of “dissemination” *M. tuberculosis* mutants

M. tuberculosis has the ability to invade and persist inside the cells other than macrophages (69). Disruption of *hbhA* gene which encodes heparin-binding hemagglutinin (HBHA) demonstrated the importance of this protein in mediating adherence and entry of *M. tuberculosis* into alveolar epithelial cells. *M. tuberculosis* with deletion in *hbhA* showed a severe defect in their ability to disseminate and colonize other organs (70).

Several transcriptional studies revealed the importance of differential expression of iron-, carbon- and oxygen responsive genes in the lungs of chronically infected mice as well as in tuberculosis patients confirming the genetic mutation studies described above (71-73). In the presence of Th1 mediated immunity, *M. tuberculosis* changed the gene expression profile to adapt to iron limitation, glucose starvation, fatty acid substrates and hypoxia (71-73). Collectively these studies provide evidence for changes in *M. tuberculosis* transcription pattern that are characteristic of non-replicating persistence (73).

2.3. Host immune response against *M. tuberculosis* infection

2.3.1. Risk of tuberculosis infection

In epidemiological studies the risk of acquiring *M. tuberculosis* infection, and the risk of developing tuberculosis disease are often considered separately. The risk of acquiring *M. tuberculosis* infection is determined by the probability of being exposed to *M. tuberculosis* and the probability of having productive infection, if exposed to *M. tuberculosis* (Figure 2).

Similarly, the risk of having tuberculosis disease consists of risk of primary tuberculosis, if infected and risk of reactivation, if latently infected (Figure 2). There is also an additional risk of re-infection (74-77).

The risk of exposure to *M. tuberculosis* within general US population is very low; it is estimated to be 0.02% - 0.08% per year (78, 79). However, the risk of exposure to TB increases if persons work and live in congregate settings such as hospitals (11.7%), prisons (27%), nursing homes (5%) or homeless shelters (18 – 70%) (80-85).

Currently there are no epidemiological studies which with a great degree of certainty can determine what the risk of infection is, if an individual is exposed to *M. tuberculosis*. It will depend on several factors, namely ability of infected person to transmit *M. tuberculosis*, the biological fitness of *M. tuberculosis*, and susceptibility of the exposed individuals (86). The ability of infected person to transmit *M. tuberculosis* will depend on the extent of pulmonary disease, and the concentration of tubercle bacilli in their sputum. There is no genetic evidence yet to demonstrate that certain *M. tuberculosis* strains are more transmissible than others. Our laboratory and others are addressing this question in the household contacts of index cases with active tuberculosis in Uganda. The susceptibility of exposed individuals will depend on two aspects, namely whether exposed individual has an underlying immunodeficiency such as HIV infection, or whether exposed individual had prior TB infection, BCG vaccination or infection with environmental mycobacteria (86).

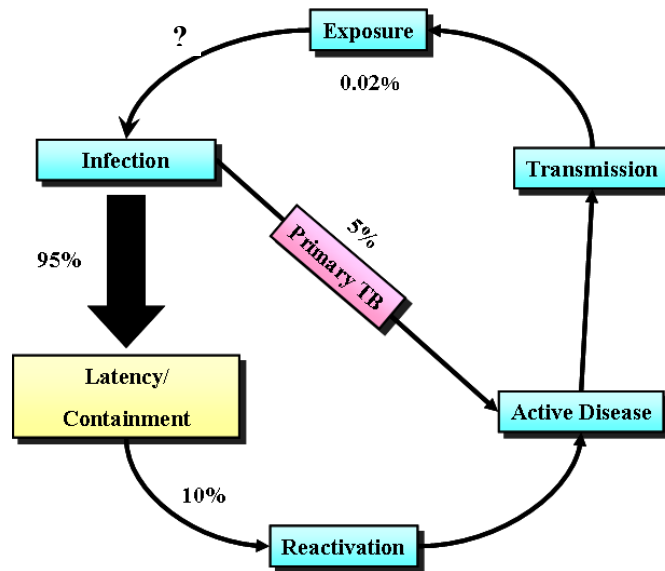


Figure 2 Transmission cycle of *M. tuberculosis*

The risk of being exposed to *M. tuberculosis* infection is low in general population (0.02 – 0.08%). Currently, there are no epidemiological data that can determine with a great degree of confidence how many of exposed individuals acquired *M. tuberculosis* infection. If infected with *M. tuberculosis*, about 5% of infected individuals will develop primary tuberculosis, while 95% of infected persons mount a strong and protective immune response leading to the containment of infection (latency). However, *M. tuberculosis* is not completely eliminated, and a risk of reactivation in latently infected people is considered to be 10% in the lifetime. In the case of severe immunosuppression or immunodeficiency, the risk of reactivation can increase to 10% per year.

The risk of primary disease, if infected will depend on the age of infected persons and on their immunological status. Young children, age 0 – 4 years, and HIV infected individuals have increased risk of *M. tuberculosis* infection (86). In general population, there is a 5% risk of progressing to primary tuberculosis, which is defined by developing active disease within two years of *M. tuberculosis* infection (87). Nevertheless, 95% of infected people will mount robust immune response and contain infection without any over signs of disease. These individuals are said to have latent tuberculosis.

The risk of reactivation, if latently infected is greatest in recently infected persons. The risk of reactivation is 0.31% per year within 3 - 5 years of infection, and 0.16% per year within 6

– 7 years of infection (86, 87). If infected individuals do not reactivate within the first 10 years of infection, it is estimated that the risk of reactivation is 10% in the lifetime of an individual.

2.3.2. Innate immunity

2.3.2.1. Role of TLR2 and TLR4 in *M. tuberculosis* recognition

Purified *M. tuberculosis* derived components such as 19-kDa lipoprotein, PIM, and LAM were all found to interact with TLR2, and depending on the ligand, induced pro- or anti-bacterial effects on antigen presenting cells. For example, 19-kDa lipoprotein induces host cell apoptosis, inhibits MHC Class II processing machinery, stimulates murine and human macrophages to secrete TNF and NO and induces IL-12 production by monocyte derived dendritic cells (reviewed in (88)). Furthermore, it was shown that both viable and killed *M. tuberculosis* activated murine macrophages expressing TLR2 and TLR4, although mycobacterium-derived TLR4 ligands are yet to be identified (88).

Considering that TLRs play an important role in pathogen recognition, it was surprising to see that TLR2^{-/-} and TLR4^{-/-} mice were fully capable of controlling *M. tuberculosis* infection. Following infection of TLR2^{-/-} and TLR4^{-/-} mice with a low dose of *M. tuberculosis* (100 CFU), both mutant strains were as resistant as wild-type control mice (89, 90). Granuloma formation, macrophage activation, and secretion of pro-inflammatory cytokines in response to low-dose infection were identical between the mutant and wild-type mice (89). In two independent studies, it was shown that infection of TLR2^{-/-} mice with a high dose of *M. tuberculosis* (500 CFU and 2000 CFU) resulted in a susceptible phenotype (89, 90). These findings suggest that paradoxically TLR2 may be important after high dose challenge during the

chronic stage of infection. In contrast, TLR4^{-/-} mice were not more susceptible than wild-type mice after high dose *M. tuberculosis* infection (2000 CFU) (89). Ongoing studies with respect to the involvement of TLR4 in protective responses against *M. tuberculosis* yielded confusing results. One study reported that macrophage recruitment and the proinflammatory response to *M. tuberculosis* were impaired in TLR4^{-/-} mice and that TLR4 signaling was required to mount protective response during chronic infection (91). In contrast, two separate studies demonstrated that TLR4^{-/-} mice were resistant to infection with *M. tuberculosis* (89, 92).

Infection of mice deficient in the myeloid differentiation factor 88 (MyD88) with *M. avium* resulted in increased susceptibility and MyD88^{-/-} mice succumbed to infection between 9 and 14 weeks (93). Interestingly, this phenotype was not reproduced after infection of TLR2^{-/-} and TLR4^{-/-} mice with *M. avium*, suggesting that other TLRs may play a protective role against mycobacterial infections (93). Two studies reported the outcome of *M. tuberculosis* infection in MyD88^{-/-} mice. Both studies demonstrated that infection of MyD88^{-/-} mice with virulent *M. tuberculosis* resulted in higher bacterial burden and more severe pathology (94, 95). However, while Sugawara *et al.* reported that expression levels of IL-12, TNF- α and IFN- γ in MyD88^{-/-} mice were comparable to wild-type mice (95), Scanga *et al.* results indicate that susceptibility of MyD88^{-/-} mice was attributed to reduced Th1 cytokines and NOS2 induction (94). Discrepancies in the two studies could be partly attributed to the use of different mouse genetic backgrounds and different strains of *M. tuberculosis*. Therefore, further studies are required to clarify the involvement of TLR signaling as a part of innate immunity in the subsequent induction of protective responses against *M. tuberculosis*.

2.3.2.2. Macrophages

After deposition in the alveolar spaces of the lung, tubercle bacilli encounter alveolar macrophages, which represent the first arm of defense. Initially they serve as hosts for bacterial replication, and secrete IL-1, IL-6 and TNF- α , which help recruit monocytes, neutrophils and lymphocytes to the site infection. In this early stage, macrophages have a limited ability to restrain bacterial growth until cell mediated immunity is induced. Recognition of infected macrophages by antigen specific T cells leads to secretion of IFN- γ and TNF- α , which synergistically activate effector mechanisms within infected macrophages (Figure 3A). The effector mechanisms of macrophages in controlling *M. tuberculosis* growth include induction of apoptosis, phagolysosomal fusion (discussed in Section 1.2.1.), oxidative burst, and NO-dependent and NO-independent mechanisms.

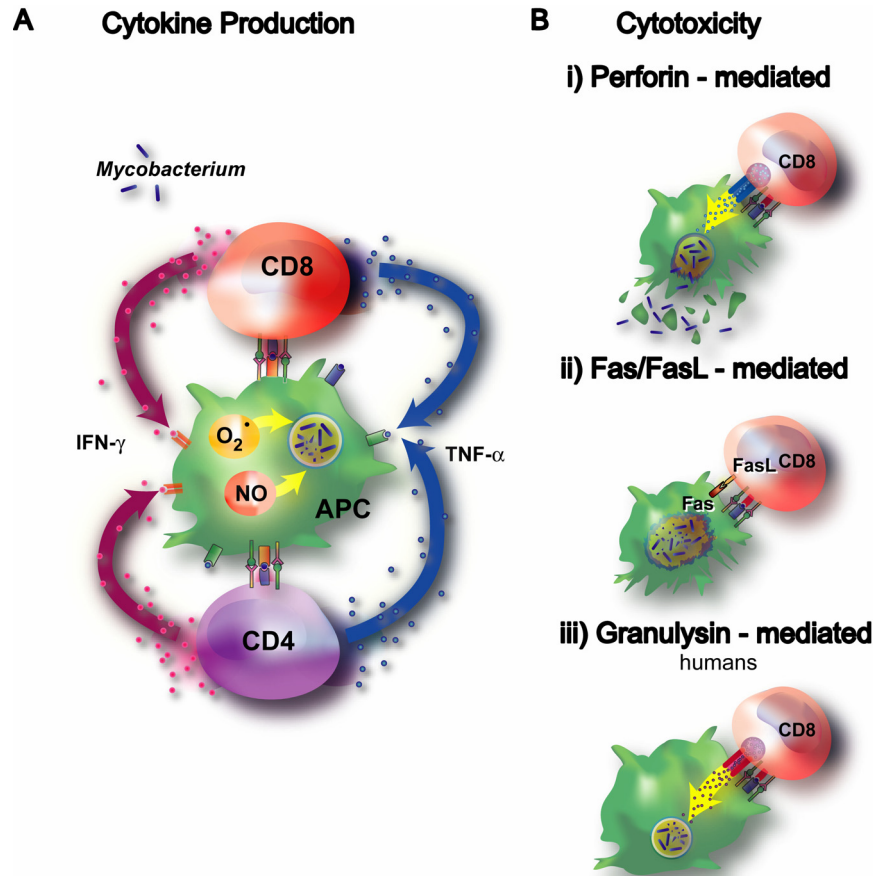


Figure 3 Innate and acquired immunity against *M. tuberculosis*

- A) Cytokine production.** Both CD4⁺ and CD8⁺ T cells are important producers of IFN- γ and TNF- α during *M. tuberculosis* infection. Both cytokines act synergistically to activate *M. tuberculosis*-harboring macrophages. Activation of macrophages results in upregulation of inducible nitric oxide synthase (NOS2), which leads to production of reactive nitrogen intermediates (RNI), such as nitric oxide (NO). RNI, together with reactive oxygen intermediates (O₂[•]), exerts anti-mycobacterial effects, which leads to reduction in viable mycobacteria.
- B) Cytotoxicity**
- (i) **Granule-dependent exocytosis pathway.** Upon recognition of *M. tuberculosis*-infected cells, CD8⁺ T cells release perforin-containing granules. Entry of effector molecules such as granzyme A and granzyme B (serine proteases) lead to apoptosis or lysis of the target cell. The lysis of unresponsive macrophages infected with *M. tuberculosis* releases the pathogen into the extracellular environment to be taken up by freshly activated macrophages, which are better equipped for killing them.
 - (ii) **Fas/FasL-mediated cytotoxicity.** Cross-linking of FasL (expressed on activated CD4⁺ and CD8⁺ T cells) and Fas (expressed on target cell) leads to recruitment of Fas Associated Death Domain (FADD) and activation of caspase 8 leading to apoptosis of target cell.
 - (iii) **direct microbicidal activity.** Granules within human CD8⁺ T cells contain a newly identified molecule, granulysin, which has direct microbicidal effect on intracellular bacteria. A murine homologue of granulysin has not been identified, and may not exist.

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Induction of apoptosis

Exposure of *M. tuberculosis* infected macrophages to extracellular ATP results in bacterial killing. This killing was dependent on an increase in cytosolic Ca^{2+} , which was linked to promotion of phagosome-lysosome fusion (96). *In vitro* studies with P2X₇^{-/-} murine macrophages demonstrated that stimulation of phagosome-lysosome fusion and ATP-mediated killing of intracellular mycobacteria was dependent on activation of P2X₇ receptor (97). In a recent study a single nucleotide polymorphism (*1513C* allele) in the human P2X₇ gene was identified (98). The change results in a single amino acid substitution at position 496 (D → A) and renders P2X₇ inactive without affecting its cell surface expression (99). The frequency of *1513C* allele is 0.12 in the Caucasian population with the prevalence of homozygous condition in 1-2% population (98). In contrast to wild-type macrophages, activation of macrophages isolated from individuals homozygous for *1513C* failed to induce apoptosis and did not result in ATP-mediated mycobacterial killing (98). These results demonstrate complete loss of killing in response to ATP suggesting that the single nucleotide polymorphism may allow for increased survival of *M. tuberculosis* in homozygous individuals.

Oxidative burst

Catalytic action of the oxidative burst by NADPH-oxidase complex produces ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^\cdot) which may be directly toxic to intracellular bacteria (Figure 3A). The role of ROS in protection against *M. tuberculosis* is questionable. The strongest evidence for the contribution of oxidative burst to macrophage effector mechanisms should come from individuals or mice that lack phox enzymes. Epidemiological evidence involving subjects with chronic granulomatous disease (CGD), who

lack one of the four subunits of NADPH-oxidase complex, indicates that there is no increased incidence of tuberculosis in these patients (100). However, tuberculosis appears to be a serious health issue for CGD patients who live in areas endemic for TB (101). Gp91^{phox}^{-/-} and p47^{phox}^{-/-} mice showed a defect at restraining *M. tuberculosis* replication, but only in the lung, temporarily, and to a small degree (102, 103). Phox^{-/-} NOS2^{-/-} double knockout mice did not die any faster following aerosol challenge with *M. tuberculosis* infection than NOS2^{-/-} mice suggesting that oxidative burst does not appear to be a major nonredundant mechanism of host defense against *M. tuberculosis* (104).

Role of reactive nitrogen intermediates in the control of M. tuberculosis infection

NO and RNI have the potential to modify and damage bacterial DNA, proteins and lipids intracellularly and extracellularly (Figure 3A). In the murine model of tuberculosis, NO plays an important and essential role in the killing of intracellular mycobacteria during acute and latent infection. NOS2^{-/-} mice were more susceptible to aerosol infection with *M. tuberculosis* than wild-type mice (105). Administration of NOS inhibitor, aminoguanidine, led to the reactivation of latent tuberculosis, suggesting that RNI pathway is at least partly responsible for preventing reactivation of latent tuberculosis in mice (46). In contrast to murine studies, there is more controversy about the role of NO in human tuberculosis. However, there is accumulating evidence now that human macrophages produce NO following *M. tuberculosis* infection (106-108). There was increased NOS2 expression in the alveolar macrophage from TB patients, and moreover NOS2 was catalytically active (106). Patients with active TB exhaled more NO than healthy controls, and the output of NO increased after administration of aerosolized IFN- γ to

patients with pulmonary TB (109, 110). Collectively these studies implicate NO as being an essential component of human defense against *M. tuberculosis*.

NO-independent mechanisms

In initial studies it was noted that NOS2^{-/-} mice survived significantly longer the aerosol challenge with virulent *M. tuberculosis* than IFN- γ ^{-/-}, IFN- γ R^{-/-}, or STAT1^{-/-} mice. These findings demonstrated the existence of IFN- γ -dependent, but NOS2-independent mechanisms against *M. tuberculosis*. MacMicking *et al.* identified LRG-47, a member of 47-kilodalton (p47) guanosine triphosphatase family, as being an important component of protective immunity against *M. tuberculosis* functioning independently of NOS2 (111). LRG47^{-/-} mice failed to control *M. tuberculosis* infection, where the defective killing by LRG47^{-/-} macrophages was attributed to the impaired fusion of lysosomes with mycobacteria containing phagosomes in response to IFN- γ signaling (111).

2.3.2.3. Dendritic cells

In the lungs of uninfected mice, CD11c⁺ cells are distributed widely in airway epithelia and within the interstitial spaces of the alveoli, suggesting that these cells are strategically placed for encounter with invading organisms (112). In the absence of infection, these cells have immature phenotype CD11c^{mid to high}, MHC Class II^{low}, CD34^{neg}, CD14^{neg}, CD8 α ^{neg}, CD11a^{hi}, CD11b^{hi} and CD54⁺ (112). Recent studies have shown that *M. tuberculosis* infects human dendritic cells via ligation of DC-SIGN by lipoarabinomannan (113, 114). Infection of both human and murine dendritic cells with *M. tuberculosis* results in maturation of these antigen presenting cells characterized by increased expression of adhesion, co-stimulatory and antigen-

presenting molecules (ICAM-1, CD54, CD40, B7.1, B7.2, MHC Class I and MHC Class II) as well as in production of inflammatory cytokines such as TNF- α , IL-1 and IL-12 (115, 116). Dendritic cell maturation can be stimulated by *M. tuberculosis* lipopeptides via TLR2 signaling pathway (117). Pre-incubation of immature human dendritic cells with anti-TLR2 blocking antibody before addition of *M. tuberculosis* lipopeptides blocked the phenotypic and functional changes associated with dendritic cell maturation (117).

The fate of *M. tuberculosis* inside murine and human dendritic cells is poorly understood. While resting murine dendritic cells supported replication of intracellular mycobacteria to a similar extent as resting macrophages, unactivated human dendritic cells appear not to be permissive for the growth of intracellular mycobacteria (116, 118). The global maturation-induced reduction in endocytosis by human dendritic cells was responsible for depriving *M. tuberculosis* of essential nutrients and stalling its growth (118). Although the fate of *M. tuberculosis* inside activated dendritic cells has not been investigated by Tailleux and colleagues, Bodnar *et al.* demonstrated that unlike activated macrophages, dendritic cells were unable to kill intracellular *M. tuberculosis* after IFN- γ and LPS stimulation (116). The ability of *M. tuberculosis* to survive, but not replicate, within activated dendritic cells, may be beneficial in the priming of T cell responses in the lymph nodes. A constant supply of secreted antigens from viable mycobacteria may induce better CD4⁺ and CD8⁺ T cell responses against tuberculosis than killed bacteria (116).

Direct comparison of dendritic cells and macrophages after *M. tuberculosis* infection revealed that dendritic cells are the primary cells involved in the priming of *M. tuberculosis*-specific acquired immunity (119). Bhatt *et al.* demonstrated that only dendritic cells receiving inflammatory stimuli from *M. tuberculosis* up-regulated expression of CCR7, and migrated to

the regional lymph nodes where they primed naïve T cells (119). Macrophages and dendritic cells respond differently to *M. tuberculosis* infection and hence have different consequences for the development of T cell responses (120). Infection of dendritic cells with *M. tuberculosis* stimulates secretion of IFN- γ inducing cytokines, such as IL-12 and IFN- α ; in contrast, macrophages respond to *M. tuberculosis* with IL-18 and IL-10 production (120, 121). In one study, IFN- γ production by *M. tuberculosis* infected macrophages was reported, suggesting that macrophages could be a significant source of Th1 cytokines, a finding that definitely merits further investigation (122). From these studies it was postulated that following *M. tuberculosis* infection, dendritic cells are involved in the induction of antimycobacterial T cell responses while macrophages modulate Th1 response within granulomas (119-121).

In addition to producing Th1/IFN- γ inducing cytokines, IFN- $\alpha\beta$ and IL-12, *M. tuberculosis* infected dendritic cells expressed CCL3, CCL4, CXCL9 and CXCL10, which were involved in the stimulation of NK and T cell migration (123). This increase in the expression of chemokines was mediated by IFN- α production as neutralization of IFN- α significantly reduced the chemotactic properties of the supernatants from *M. tuberculosis* infected dendritic cells (123). Therefore, dendritic cell-derived IFN- α may contribute to recruitment and homing of activated effector cells to the site of infection.

Collectively, these studies underscore the importance of dendritic cells in the initiation of protective Th1 response against *M. tuberculosis*. The fact that *M. tuberculosis* infection is usually contained but not eliminated by the host, indicates that *M. tuberculosis* has developed evasive mechanisms by which it suppresses the robustness of immune response generated against it. *M. tuberculosis*-derived LAM binds to DC-SIGN, an important receptor on dendritic cells for mycobacteria, blocks maturation of *M. tuberculosis*-infected dendritic cells by interfering with

TLR signaling, and induces the production of immunosuppressive cytokine IL-10 (113). These findings indicate that in humans, *M. tuberculosis* may target DC-SIGN to suppress cellular immune responses and induce the state of antigen-specific tolerance (113).

2.3.2.4. NK cells

Murine NK cells respond to pulmonary infection with *M. tuberculosis* during the first 21 days by increasing in numbers, expressing activation and maturation makers, producing IFN- γ and perforin (124). However, depletion of NK cells did not influence bacterial load or disease progression in the lungs, suggesting that NK cells are not essential for the host resistance to *M. tuberculosis* (124). In humans, NK cells were found to regulate CD8⁺ T cell effector function at multiple levels. Depletion of NK cells from PBMC of healthy PPD⁺ individuals resulted in the decreased frequency of IFN- γ producing and cytolytic CD8⁺ T cells (125). NK cells maintained the frequency of IFN- γ producing CD8⁺ T cells by stimulating monocytes to produce IL-15 and IL-18 (125). The lytic function of CD8⁺ T cells appeared to be regulated by the direct contact between the CD40L on NK cells and CD40 on infected monocytes (125). Thus, NK cells appear to bridge innate and adaptive responses in protective immunity against *M. tuberculosis*.

2.3.3. Acquired immunity

2.3.3.1. CD4⁺ T cells

Mice deficient in CD4⁺ T cells either through deletion of genes for CD4, MHC Class II or CIITA molecules, are susceptible to *M. tuberculosis* acute infection (126, 127). MHC Class II^{-/-} and CD4^{-/-} mice had diminished production of IFN- γ in the first three weeks of infection. By four weeks post-infection, CD4-deficient mice had wild-type levels of IFN- γ due to the compensatory increase in IFN- γ producing CD8⁺ T cells (126). However, MHC II ^{-/-} mice were not rescued, and they succumbed to infection with mean survival time of 41 days (126). The importance of CD4⁺ T cells in chronic *M. tuberculosis* infection was demonstrated using an anti-CD4 antibody mediated depletion model. In this study, mice were infected with a low dose of *M. tuberculosis*, and at six months post-infection were treated either with anti-CD4 antibody or isotype control for the duration of the study (48). Antibody-mediated depletion of CD4⁺ T cells resulted in rapid reactivation of a persistent infection, with dramatically increased bacterial numbers in multiple organs, increased pathology in the lungs and decreased survival. Interestingly, IFN- γ and NOS2 gene and protein expression was comparable to wild-type mice at all time points. These findings indicate that CD4⁺ T cells are absolutely essential for the control of acute infection, and for preventing reactivation of persistent tuberculosis in the murine model. Furthermore, CD4⁺ T cell protective function extends beyond IFN- γ production and macrophage activation. In humans, the AIDS epidemic demonstrated that loss of CD4⁺ T cells greatly increases the risk of acquiring primary tuberculosis, and the risk of reactivation of latent tuberculosis is increased to 10% per year within HIV positive population (128).

How do CD4⁺ T cells contribute to protection against M. tuberculosis?

Indisputably, the most important role of CD4⁺ T cells in protective immunity against *M. tuberculosis* is IFN- γ production, which will lead to NOS2 expression and activation of infected macrophages (Figure 3A). Since CD4⁺ T cells are the most important source of IFN- γ , delayed production of this important cytokine rendered CD4^{-/-} and MHC II^{-/-} mice susceptible to *M. tuberculosis* induced disease (126). These results indicate that the CD4⁺ T cell contribution to the IFN- γ production, macrophage activation and granuloma formation early in infection is indispensable and cannot be replaced by other immune cells, such as NK and CD8⁺ T cells. However, CD4⁺ T cells have other IFN- γ and NOS2-independent mechanisms of protection, which are yet to be identified. It is these mechanisms that contribute to the role of CD4⁺ T cells in preventing reactivation of persistent infection in mice. Despite the wild-type levels of IFN- γ and NOS2, chronically infected mice rapidly reactivated after short-term CD4 depletion and succumbed to *M. tuberculosis* infection (48).

Cowley *et al.* attempted to identify IFN- γ independent mechanism of CD4 mediated immunity. In an *in vitro* model CD4⁺ T cells were responsible for >90% inhibition of intracellular *M. tuberculosis* growth in the complete absence of IFN- γ signaling (129). This CD4⁺ T cell-mediated, IFN- γ independent, control of mycobacterial replication was dependent largely on NO (129). The signals delivered by CD4⁺ T cells to the infected macrophages could be dependent on CD40/CD40L interaction; however, results from this thesis indicate that CD40^{-/-} macrophages were equally capable of killing intracellular mycobacteria following IFN- γ /LPS stimulation or activation with *M. tuberculosis* specific T cells (Chapter 1). Although these results suggests that CD40 ligation on macrophages is not necessary for macrophage activation, it is possible that in the absence of IFN- γ signaling, CD40/CD40L interaction may be a major

pathway for macrophage activation. Another promising candidate is TNF- α as neutralization of TNF- α significantly diminished the ability of macrophages to control intracellular *M. tuberculosis* infection (129).

In addition to cytokine production which has been demonstrated in *M. tuberculosis* infected humans and mice (130-134), human CD4⁺ T cells also express perforin and contribute to the killing of infected macrophages in both granule exocytosis dependent and Fas/FasL dependent pathways (135-137). In contrast, murine CD4⁺ T cells do not express perforin, but they are required for the normal development of cytotoxic CD8⁺ T cells responses during acute *M. tuberculosis* infection (138).

The role of CD4⁺ T cells in preventing reactivation of latent tuberculosis could be associated with their role in maintaining organized granuloma structure. Depletion of CD4⁺ T cells from the chronically infected mice could result in the loss of structural integrity of the tuberculous granuloma, thus leading to TB reactivation (48).

2.3.3.2. CD8⁺ T cells

The evidence for an essential role of CD8⁺ T cells is not quite as compelling. For years many researchers in the tuberculosis field ignored this T cell subset. Since *M. tuberculosis* lives primarily within a vacuole inside the cell, rather than in the cytoplasm, it seemed unlikely that antigens would be effectively presented to CD8⁺ T cells by MHC Class I molecules.

However, early studies using antibody-mediated T cell subset depletion suggested that CD8⁺ T cells, in addition to CD4⁺ T cells, were necessary for control of *M. tuberculosis* infection (139) and adoptive transfer of purified immune CD8⁺ T cells reduced the numbers of *M. tuberculosis* bacteria in the spleens of infected mice, albeit at a lower efficiency compared to

CD4⁺ T cells (140, 141). The development of gene-disrupted mice provided stronger data for a role of CD8⁺ T cells in the control of *M. tuberculosis* infection.

Mice genetically deficient in β_2 -microglobulin (β_2m), which lack functional MHC Class I molecules and consequently CD8⁺ T cells, failed to control infection, particularly in the lung, and succumbed prematurely to tuberculosis (142). Although it was reasonable to assume at the time (10 years ago) that susceptibility of $\beta_2m^{-/-}$ mice was due to a drastically reduced total number of peripheral CD8⁺ T cells, the defect in $\beta_2m^{-/-}$ mice is broad. In addition to the absence of *classical* MHC Ia molecules, which present peptides to CD8⁺ T cells, these mice also lack functional CD1 and other *non-classical* MHC Ib molecules, which present lipid antigens and N-formylated peptides derived from bacteria. Thus, even though CD8⁺ T cells were shown to be important for control of infection, the molecules used to present antigens to the protective T cell subset remained unclear.

To determine relative contributions of classical or non-classical MHC Class I-dependent CD8⁺ T cell populations in protection against tuberculosis, a series of gene-disrupted mouse strains were compared for susceptibility to intravenous *M. tuberculosis* infection, as measured by survival time and bacterial loads. Among the strains tested, the most susceptible mice were the $\beta_2m^{-/-}$, followed by TAP1^{-/-} (transporter associated with antigen processing), followed by CD8 $\alpha^{-/-}$, perforin^{-/-}, and CD1d^{-/-} mice (143). The conclusion was that classically restricted (which are TAP1-dependent) CD8⁺ T cells contribute to *in vivo* protection against *M. tuberculosis*, however, the role of CD8⁺ T cells in protective immunity was not limited to perforin-dependent cytotoxicity (143).

It is well established that the CD4⁺ T cell response is crucial to control infection, and it must be targeted in vaccine development. While many investigators believe that CD8⁺ T cells are

important and should be considered in design of new vaccines, others are not as convinced. In a recent study, wild-type and gene-knockout mice deficient in both CD4⁺ and CD8⁺ T cells, MHC I (lack CD8⁺ T cells) or MHC II (lack CD4⁺ T cells) were infected via aerosol and monitored for survival and ability to control infection (144). The conclusion of this study was that in contrast to CD4⁺ T cells, CD8⁺ T cells were dispensable, and not essential for the control of infection. However, the data from this study do not fully support these conclusions. Although mice devoid of CD4⁺ T cells died earlier from tuberculosis than CD8⁺ T cell-deficient mice, the absence of both CD4⁺ and CD8⁺ T cells resulted in even greater susceptibility. One interpretation is that the presence of CD8⁺ T cells in CD4⁺ T cell-deficient mice resulted not only in increased survival time, but also prevented excessive immunopathology when compared to mice without both T cell subsets (144). The fact that mice devoid of CD8⁺ T cells still succumb to *M. tuberculosis* infection despite the development of fully functional CD4⁺ T cell responses argues that CD8⁺ T cells may play an important role in controlling chronic infection. Furthermore, depletion of CD8⁺ T cells resulted in reactivation of latent tuberculosis in a murine model, suggesting that this T cell subset may also be essential for controlling latent tuberculosis (145).

How do CD8⁺ T cells contribute to protection against M. tuberculosis?

CD8⁺ T cells have the potential to affect antimycobacterial immunity in a number of ways. These cells may function as a source of Type 1 cytokines such as IFN- γ and TNF- α or they may exert their protective effect by killing infected macrophages within the tissues (Figure 3A and 3B). Results from experiments using gene-deficient mice have generated controversy about which CD8⁺ T cell-mediated mechanisms are responsible for protection against tuberculosis. Intracellular cytokine staining indicated that comparable numbers of activated CD4⁺

and CD8⁺ T cells in the lungs of infected mice were primed to produce IFN- γ after brief, non-specific stimulation (130). However, intracellular cytokine staining of unstimulated CD4⁺ and CD8⁺ T cells from the lungs of infected mice, which is more reflective of an *in vivo* situation, suggested an important difference between the two T cell subsets. Early in infection at least 13% of CD4⁺ T cells produced IFN- γ directly *ex vivo*, in contrast to <5% of IFN- γ producing CD8⁺ T cells (130). The presence of large numbers of activated CD8⁺ T cells in the lungs, with moderate cytokine production, suggests that the cytotoxic functions of CD8⁺ T cells may be important in the response against acute *M. tuberculosis* infection (130).

Early in infection, *M. tuberculosis*-specific CD8⁺ T cells from the lungs expressed perforin *in vivo*, and lysed *M. tuberculosis*-infected macrophages in a perforin- and MHC Class I-dependent manner (146). Additional evidence for the importance of CD8⁺ cytotoxic effector functions comes from studies in CD4⁺ T cell-deficient mice, which succumbed to *M. tuberculosis* acute or chronic infection despite compensatory IFN- γ production by CD8⁺ T cells resulting in wild-type levels of this cytokine in the lungs (48, 126). Subsequently, it was shown that CD8⁺ T cells from CD4⁺ T cell-deficient mice have impaired cytotoxic function in the lungs of *M. tuberculosis* infected mice (138). These results suggest that the susceptibility to tuberculosis seen in CD4⁺ T cell-deficient mice may be partly due to defective cytotoxic effector functions of CD8⁺ T cells. Furthermore, the level of protection against *M. tuberculosis* conferred by the adoptive transfer of CD8⁺ T cell clones into recipient mice correlated with the level of cytotoxicity rather than with the level of IFN- γ secretion (147).

On the other hand, several studies suggest that cytokine secretion may be the major effector function of CD8⁺ T cells. In experiments using IFN- γ -/- mice as CD8⁺ T cell donors, production of IFN- γ was required for CD8⁺ T cells to exert a modest anti-mycobacterial effect in

CD4⁺ T cell deficient mice (148). Moreover, mice with targeted disruptions in the genes for Fas, perforin or granzyme were no more susceptible to acute infection with *M. tuberculosis* than their wild-type littermates (149, 150). The fact that perforin^{-/-} mice succumb later in infection suggests that perforin-mediated cytotoxic activity of CD8⁺ T cells may be more important during the chronic stage of infection (143). However, interpretation of these results is complicated by the fact that perforin gene disruption is associated with a compensatory activation of T cells, and expression of increased levels of cytokines even in the absence of experimental infection (150, 151). Perforin deficiency also affects the function of CD8⁺ T cells during acute *M. tuberculosis* infection (146). Perforin^{-/-} mice had increased numbers of CD8⁺ T lymphocytes, which were in a state of hyperactivation with 4.5-fold increased IFN- γ production compared with wild-type mice (146). This could mask any effect of a lack of perforin on control of the infection.

There is one confounding factor for addressing the importance of CD8⁺ T cells in control of *M. tuberculosis* in the murine model. Recent studies demonstrated that human CD8⁺ T cells recognizing *M. tuberculosis*-infected macrophages had the ability to directly kill intracellular mycobacteria (152) (Figure 3B). This killing was due to a granule-associated protein, granulysin (153). The purified molecule was toxic to mycobacteria, but required perforin pore formation to enter an infected cell. This was an important demonstration of how CD8⁺ T cells could be playing a direct role in control of *M. tuberculosis* infection. Unfortunately, mice do not have a granulysin homolog, and at this time it is not possible to test the true contribution of this mechanism to control of infection in the mouse model. Therefore, data from murine studies suggesting that CD8⁺ T cells are not necessary for control of infection must take into account the absence of what may be the key mechanism by which CD8⁺ T cells participate in anti-tuberculosis immunity. Questions regarding CD8⁺ T cell induction, kinetics of migration to the

lungs, and elucidation of CD8⁺ T cell effector mechanisms that contribute to protective immunity against tuberculosis are a subject of investigation in the chapter 2 of this thesis.

2.3.3.3. B cells

Although antibodies against major *M. tuberculosis* proteins, such as ESAT-6, PPE antigen, and Des protein, have been detected in the sera of *M. tuberculosis*-infected patients (154-156), the role of B cells and antibodies in protective immunity against *M. tuberculosis* has been difficult to define. A review of the published literature on the role of antibody-mediated immunity against *M. tuberculosis* over the past 100 years presented evidence for and against protective effects of antibody therapy (157). The major problems with the early studies from 1888 – 1920 were that there were no consistent serum formulations, no appropriate controls and no detailed diagnostic evaluations following antibody treatment (157). Several modern studies (after 1930s) revealed that there was a correlation between antibody titers and improved outcome of tuberculosis and the efficacy of treatment depended on the target antigen (158-161). In contrast, other studies reported no association between the presence of serum antibodies and outcome of tuberculosis infection (162-164). Results from murine studies using B cell knockout mice revealed equally contradictory results. In one report B cells contributed very little or not at all to protective immunity in a murine model of tuberculosis, as B cell-deficient mice did not show significant differences in cytokine mRNA expression, pathology or bacterial burden in multiple organ during acute infection with a low dose of *M. tuberculosis* (165). In contrast, Bosio *et al.* reported a potential role of B cells in pulmonary granuloma formation and subsequent dissemination of *M. tuberculosis* in wild-type mice (166). Collectively, these studies call for methodical evaluation of the role of B cells and antibody-mediated immunity against *M.*

tuberculosis. Use of monoclonal antibodies to defined antigen targets may finally shed light on the efficacy of individual antibodies in modifying the course of *M. tuberculosis* infection (157).

2.3.4. Cytokines

Although the roles of many cytokines in protective immunity against *M. tuberculosis* have not been explored, the roles of IL-12, IFN- γ , TNF- α are well established; while, the influence of IL-10 on the course of *M. tuberculosis* infection has been more difficult to demonstrate.

2.3.4.1. IL-12

The absence of the bioactive IL-12 results in unrestrained growth of *M. tuberculosis*. The inability of IL12p40^{-/-} mice to control bacterial growth was associated with the absence of both innate and acquired sources of IFN- γ (167). However, the severity of the susceptible phenotype was more pronounced in IL12p40^{-/-} than in IL12p35^{-/-} mice suggesting that mice lacking bioactive IL-12 can generate antigen specific IFN- γ responses if the IL12p40 is present (168, 169). Mice lacking p35 subunit exhibited moderate ability to control bacterial growth and increased survival, which were associated with the induction of antigen-specific IFN- γ responses (168, 169). A candidate molecule of inducing protective response in IL12p35^{-/-} mice is IL-23, which is composed of p40 and p19 subunits. Although a direct role for IL-23 in protective immunity against *M. tuberculosis* has not been investigated yet, expression of p19 subunit was demonstrated in the lungs of *M. tuberculosis* infected mice (168, 169). IL-12 increases resistance of susceptible BALB/c and CD4⁺ T cell-deficient mice, although the modulatory effects of IL-12, which lead to better protection, have not been clearly defined (170, 171). In IL-12 treated

CD4^{-/-} mice, the early increase in IFN- γ , most likely due to activated NK cells, could contribute to the increased survival of *M. tuberculosis*-infected CD4^{-/-} mice (171).

Expression of IL12R β 1 and IL12R β 2 correlated well with the amount of IFN- γ production in human tuberculosis. Patients with active tuberculosis had reduced frequency of IFN- γ producing T cells and expressed low levels of IL12R β 1 and IL12R β 2 mRNA when compared to healthy PPD⁺ individuals (172). Addition of neutralizing anti-IL-10 and anti-TGF- β antibodies to *M. tuberculosis*-stimulated PBMC from tuberculosis patients increased expression of IL12R β 1 and IL12R β 2 subunits and enhanced IFN- γ production, suggesting that production of immunosuppressive cytokines in TB patients may reduce T cell responsiveness to IL-12 and result in weak IFN- γ responses (172).

2.3.4.2. IFN- γ

IFN- γ ^{-/-} mice are unable to control infection with virulent *M. tuberculosis*, and are the most susceptible mouse strain tested to date. Although granuloma formation was not impaired in the absence of IFN- γ , activation of macrophages was defective leading to unrestrained mycobacterial replication and widespread necrosis throughout the major target organs (173, 174). Direct comparison of IFN- γ ^{-/-} and NOS2^{-/-} mice revealed that NOS2^{-/-} mice lived significantly longer than IFN- γ ^{-/-} mice, which points to the existence of NO-independent, IFN- γ mediated protective mechanisms. A recent study by MacMicking *et al.* revealed that IFN- γ mediates phagosome-lysosomal fusion in infected macrophages through the action of LRG47 phosphatase (section 1.3.2.2., NO-independent mechanisms).

2.3.4.3. TNF- α

The effects of TNF- α in protective immunity against tuberculosis are pleotropic. This cytokine has been implicated in causing immunopathology, if present in elevated concentration (175), inducing apoptosis (176), controlling metalloproteinase-9 expression (177), but most importantly in orchestrating chemokine expression, thus regulating granuloma formation and maintenance of granuloma integrity (178-180).

TNFp55^{-/-} and TNF^{-/-} mice and mice in which TNF- α was neutralized succumb to acute *M. tuberculosis* infection due to delayed granuloma formation and RNI production (178, 181). Furthermore, neutralization of TNF- α in chronically infected mice led to fatal reactivation of persistent tuberculosis, increased bacterial burden, severe pathology and augmented IL-10 production, suggesting that TNF- α plays an essential role in the control of chronic *M. tuberculosis* infection (49, 182). In humans, reactivation of tuberculosis was associated with administration of Infliximab, a TNF neutralizing agent. In a study by Keane *et al.* 70 cases of tuberculosis were identified among 147,000 patients who received Infliximab for the treatment of inflammatory diseases such as rheumatoid arthritis and Chron's disease (183). In a similar study, treatment of patients with Etanercept, another commercially available anti-TNF agent, resulted in 25 cases of tuberculosis out of 121,000 patients that received the therapy (184). Collectively murine and human studies suggest that TNF is important in preventing reactivation of latent tuberculosis, and that patients receiving anti-TNF agents should be screened and treated for *M. tuberculosis* infection and closely monitored during anti-TNF therapy.

2.3.4.4. IL-10

IL-10 antagonizes the action of proinflammatory responses by downregulating expression of IFN- γ , TNF and IL-12. Cross-linking of the mannose receptor or DC-SIGN on human dendritic cells with mannosylated LAM will induce the production of anti-inflammatory cytokine such as IL-10 (113, 185). Anergy in the setting of tuberculosis refers to the absence of delayed hypersensitivity response to PPD in *M. tuberculosis* infected patients (186). It is estimated that about 15% of infected individuals will be PPD negative, and this anergy is contributed to the presence of IL-10 producing T cells, which suppress immune responses (186). Expression of IL-10 in tuberculosis patients was associated with aberrant TCR mediated signaling and reduced expression of CTLA-4 (186, 187), which led to the hypothesis that chronic *M. tuberculosis*-mediated stimulation of T cells in the absence of IFN- γ and presence of IL-10 will lead to T cell anergy and establishment of *M. tuberculosis* persistence.

The influence of IL-10 on the development of tuberculosis disease in mice is still a question of debate. Infection of IL-10^{-/-} mice with *M. tuberculosis* resulted in either a minor increase in resistance or no difference at all (188-190). Mice that overexpressed IL-10 in all cells showed no difference in bacterial burden during acute infection; however, during chronic infection there was a significant increase in bacterial numbers, which was associated with decreased mRNA production for TNF- α and IL12p40, and a decrease in antigen-specific IFN- γ responses, suggesting that IL-10 overexpression can lead to suppression of Th1 immunity (191).

2.3.5. Chemokines

Granuloma formation is a hallmark of tuberculosis. The granuloma is composed of centrally located macrophages surrounded by the cuff of T and B lymphocytes. Recent studies

indicate that infiltration of cells into infected lungs and formation of granulomas are mediated by chemokines. It has been very difficult to study roles of individual chemokines in tuberculosis due to redundancy in the system as a single cell type will express multiple receptors, and a single receptor will bind multiple ligands. Nevertheless, some common observations have been drawn regarding involvement of chemokines in *M. tuberculosis* infection.

M. tuberculosis is a potent inducer of chemokine expression (reviewed in (192)). This was demonstrated using *in vitro* macrophage system where rapid expression of CCL2, CCL3, CCL4 and CCL5 by infected macrophages was detected as early as 2 hours post-infection (180, 193). Expression of CCL2, CCL3, CCL7, CCL12, CXCL2 and CXCL10 was demonstrated following normal course of *M. tuberculosis* infection in wild-type mice (193). Although macrophages appear to be the most attractive candidate for chemokine expression, several studies reported that human bronchial epithelial cells also expressed chemokines, most notably CXCL8, CXCL9, CXCL10 and CXCL11 (194). Generation of knockout mice was essential for addressing the role of CCR2 and CCR5 ligands in controlling *M. tuberculosis* infection. Although CCR2^{-/-} mice successfully controlled low dose infection with *M. tuberculosis* they succumbed to *M. tuberculosis* following high dose challenge (195, 196). These mice were characterized by a severe defect in monocyte/macrophage migration to the inflammatory site, while T cell migration, particularly CD4⁺ T cells, was reduced only during early stages of infection (195, 196). The studies with CCR2^{-/-} mice demonstrated that macrophage infiltration into *M. tuberculosis* infected lungs was strongly mediated by CCR2 regardless of infection route, dose or strain of *M. tuberculosis* (195, 196). CCR5^{-/-} mice were also able to control *M. tuberculosis* infection with formation of histologically normal granulomas. Although there was no difference in bacterial numbers between the knockout and wild-type mice, CCR5^{-/-} were

characterized by extensive infiltration of macrophages and T lymphocytes into infected lungs, particularly during chronic infection. This dysregulation could be due to an excess of the CCR5-binding ligands, which through signaling with a different chemokine receptor, such as CCR1, will lead to increased infiltration of cells to the site of infection (Algood, H.M.S, in press).

Studies with TNF neutralizing antibodies, TNF^{-/-} or TNFRp55^{-/-} mice point to delayed granuloma formation or loss of granuloma structural integrity resulting in the susceptibility to acute infection or reactivation of latent TB. These studies suggest that TNF may orchestrate granuloma formation and maintain granuloma structural integrity through induction of chemokine expression. Our laboratory has demonstrated that neutralization of TNF in *M. tuberculosis* infected macrophages led to a reduction in expression of CCL5, CXCL9, and CXCL10 (180). In TNF-deficient mice, although cells migrated to the lungs, there was no evidence of structured granuloma formation. Most importantly, isolation of CD11b⁺ cells from the lungs of TNF-deficient and wild-type revealed significant difference in the levels of chemokine expression *in vivo*. CD11b⁺ cells from TNF-deficient mice had reduced expression of CCL5, CXCL9, CXCL10, suggesting that TNF affects chemokine expression by macrophages *in vitro* and in *in vivo* (180). Hence, this and other studies provide evidence for the role of TNF in directly controlling chemokine expression and granuloma formation in *M. tuberculosis* infection (178-180).

3. STATEMENT OF THE PROBLEM

The goal of this study was to investigate the conditions required for efficient priming and maintenance of T cell mediated immunity against *Mycobacterium tuberculosis*, and the factors necessary for generation of protective CD8⁺ T cell memory responses.

Although the importance of T cell mediated immunity is well established in controlling *M. tuberculosis* infection, very little is known about what is required to initiate a robust and protective immune response against this pathogen. Persistent exposure of T cells to high antigen dose can lead to dysfunctional T cell responses, characterized by either T cell exhaustion or deletion. It has not been studied yet how exposure to high antigen dose affects the functionality of T cells in the face of persistent *M. tuberculosis* infection.

The ultimate objective of vaccine design is induction of long-lasting protective immunity. Currently, little is known about the factors required for generation and maintenance of memory CD4⁺ and CD8⁺ T cell responses against *M. tuberculosis*.

The work presented in this thesis addresses several important questions of T cell biology in tuberculosis, namely priming of effector T cells, maintenance and regulation of effector functions during persistent infection, and establishment of protective CD8⁺ T cell memory responses in the absence of CD4⁺ T cells or IL-15. Using murine model of tuberculosis, the following hypothesis will be tested: 1) CD40 ligation is required for the priming of strong Th1 response; 2) inability of immune response to eliminate *M. tuberculosis* is a result of T cell exhaustion as a consequence of continuous antigenic stimulation in the lungs during persistent *M. tuberculosis* infection; 3) there is differential regulation of CD8⁺ T cell effector functions during different phases of immune response; 4) IL-15 is required for the maintenance of CD4⁺ and CD8⁺ T cell effector functions and for the homeostatic proliferation of CD8⁺ memory T

cells; 5) CD4⁺ T cell help is required for the generation and maintenance of functional memory CD8⁺ T cells during primary and secondary *M. tuberculosis* infection. Consequently, the following specific aims are set forward:

1. Evaluate the priming of IFN- γ T cell responses following *M. tuberculosis* infection in CD40^{-/-} and wild-type mice.
2. Examine proliferation, activation status and effector functions of CD4⁺ and CD8⁺ T cells during acute and chronic *M. tuberculosis* infection.
3. Analyze the effector functions of CD4⁺ and CD8⁺ T cells after primary and secondary *M. tuberculosis* infection of IL-15^{-/-} and wild-type mice.
4. Assess the consequences of CD4⁺ T cell depletion during priming of effector CD8⁺ T cells on the development of CD8⁺ T cell memory.
5. Evaluate the effects of CD4⁺ T cell depletion during secondary infection on the maintenance of memory CD8⁺ T cell responses.

The results from these studies will contribute to a better understanding of what encompasses protective immune response against *M. tuberculosis*, and how we can use this knowledge to improve the efficacy of current and future anti-tuberculosis vaccines.

4. CHAPTER 1 CD40, BUT NOT CD40L, IS REQUIRED FOR THE PRIMING OF *MYCOBACTERIUM TUBERCULOSIS* SPECIFIC T CELL RESPONSES

This chapter has been adapted from a published study (Lazarevic V *et al.* (2003) *Immunity* 19:823-835) with permission from Elsevier. The chapter includes additional data that was not shown in the published paper.

4.1. Introduction

CD40 is expressed on antigen presenting cells (APCs) such as B cells, macrophages (MΦ) and dendritic cells (DC) while CD40 ligand (CD40L) is primarily restricted to activated CD4⁺ T cells (197). CD40 and its ligand are essential for B cell activation, proliferation, survival, isotype switching, germinal center formation, and memory generation (reviewed in (198)). CD40L^{-/-} mice are also severely impaired in primary T cell responses to protein antigens (199). Dendritic cells constitutively express low levels of co-stimulatory molecules and require activation to become competent APCs for priming naïve T cells (200). It has been proposed that CD40/CD40L interaction is required for the induction of co-stimulatory activity on APCs (201).

Studies in CD40^{-/-} and CD40L^{-/-} mice demonstrated a critical role for CD40/CD40L interaction in the protective immune response against intracellular parasitic pathogens such as *Leishmania spp* and *Trypanosoma cruzi* (202-205). In these models, infected CD40^{-/-} and CD40L^{-/-} mice were unable to control the growth of parasites and succumbed to infection due to impaired IFN-γ production by T cells. The failure of CD40L^{-/-} mice to mount a protective Th1 response was attributed to the inability of CD40L^{-/-} T cells to induce IL-12 production by infected macrophages, even in the presence of exogenous IFN-γ (203, 204). In addition,

activation of macrophages and production of nitric oxide (NO) was not evident in infected CD40L^{-/-} mice, providing direct evidence for the role of CD40/CD40L in the effector functions of macrophages (202).

However, CD40/CD40L interaction is not required to mount a protective immune response against all intracellular pathogens. Strong activation of primary CD8⁺ T cell responses after infection of CD40L^{-/-} mice with *Listeria monocytogenes* or viruses such as lymphocytic choriomeningitis virus, Pichinde virus or vesicular stomatitis virus suggests that priming of CD8⁺ T cells can occur independently of CD40/CD40L interaction (206-209). The induction of CD40/CD40L independent immune responses against some viruses can be explained by the observation that infection of APCs with certain pathogens is sufficient to activate APCs thus bypassing the need for co-stimulation (210).

We and others have previously demonstrated that control of *Mycobacterium tuberculosis* acute and chronic infection is dependent on CD4⁺ T cells (48, 126, 144, 211). Although CD4⁺ T cells are clearly important for IFN- γ production and macrophage activation, it appears that these cells have additional roles in control of *M. tuberculosis* infection (48). Since CD4⁺ T cells are the primary source of CD40L (197), we sought to determine whether CD40L interaction with CD40 on APCs was an important function of CD4⁺ T cells in *M. tuberculosis* infection. Our initial hypothesis was that this interaction is not essential for generation of protective immune responses for several reasons: (i) infection of both human and murine dendritic cells with *M. tuberculosis* was sufficient to up-regulate cell surface expression of antigen presenting and co-stimulatory molecules as well as induce IL-12 production (115, 116), (ii) CD8⁺ T cells were primed to produce IFN- γ in the absence of CD4⁺ T cells *in vitro* and in *M. tuberculosis*-infected mice (126, 138), and (iii) CD40L^{-/-} mice were resistant to intravenous infection with *M.*

tuberculosis (212). CD4⁺ T cells from CD40L^{-/-} mice produced wild-type levels of IFN- γ when stimulated with purified protein derivative (PPD) *in vitro*, suggesting that CD40L was not necessary for priming T cells during *M. tuberculosis* infection (212).

Contrary to our expectations, CD40^{-/-} mice were susceptible to aerosol infection with *M. tuberculosis* due to poor priming of IFN- γ producing T cells in the lymph nodes of CD40^{-/-} mice as a result of attenuated IL-12 production. The subsequent deficiency in IFN- γ producing T cells in the lungs of CD40^{-/-} mice culminated in fatal, uncontrolled bacterial growth. However, CD40L^{-/-} mice were resistant to aerosol infection, confirming the results from a previously published study (212). Such asymmetry between the outcome of infection in CD40^{-/-} and CD40L^{-/-} mice points to the existence of an additional ligand for CD40. Here, we show that *M. tuberculosis* Hsp70 functions as an alternative ligand for CD40 as it induced significant IL-12 production by wild-type but not CD40^{-/-} dendritic cells. Overall, our results highlight an important role for ligation of the CD40 molecule on APC in the control of *M. tuberculosis* infection.

4.2. Materials and Methods

4.2.1. Mice

C57BL/6 mice, CD40L^{-/-} (B6.129S2-*Tnfsf5*^{tm1lmx}) mice and CD40^{-/-} (B6.129P2-*Tnfrsf5*^{tm1Kik}) breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). β 2m^{-/-}, MHC II^{-/-} and CD40^{-/-} mice were bred in the University of Pittsburgh Biotechnology Center. All mice were maintained under specific pathogen-free conditions and used at 8-12 weeks of age. The University Institutional Animal Care and Use Committee approved all animal protocols employed in the study.

4.2.2. Bacteria and infections

Aerosol and intravenous infections with *M. tuberculosis* (Erdman strain, Trudeau Institute, Saranac Lake, NY) were performed as described previously (146). In the anti-CD40L blocking study, C57BL/6 mice were injected with 250 μ g/injection of blocking anti-CD40L antibody MR1 (a generous gift from Dr. Robert Hendricks, University of Pittsburgh) prior to infection, and every other day throughout the duration of the study.

4.2.3. CFU determination

Bacterial burden was determined by plating serial dilutions of lung and spleen homogenates onto 7H10 agar plates (Difco). Plates were incubated at 37°C in 5% CO₂ for 21 days prior to counting colonies.

4.2.4. Histology and immunohistochemistry

For histological analysis, organs were fixed in 10% normal buffered formalin, embedded in paraffin, and 6 μm sections were stained with hematoxylin and eosin. For NOS2 immunohistochemistry, sections were stained with anti-NOS2 antibody (Transduction Laboratories, Cincinnati, OH) or rabbit IgG control (Accurate Chemical & Scientific Corp., Westbury, NY) as described previously (48).

4.2.5. Flow cytometry

Lung and lymph node single cell suspensions were prepared and stained as described previously (146). Cells were stained with anti-CD4 (PE; clone H129.19), anti-CD8 (Cy-Chrome; clone 53-6.7), anti-CD69 (FITC; clone H1.2F3), anti-CD40 (PE; clone 3/23), anti-CD11c (PE; clone HL3), anti-B220 (PE; clone RA3-6B2), anti-MHC Class I (H-2D^b; FITC; clone KH95), anti-MHC Class II (I-A^b; PE; clone AF6-120.1), and anti-B7.2 (PE; clone GL1) fluorescently conjugated antibodies. All antibodies were used at 0.2 $\mu\text{g}/10^6$ cells and were purchased from BD Pharmingen (San Diego, CA). Cells were collected on a FACS Caliber (Beckon Dickinson) and analyzed by CellQuest software (Becton Dickinson, Immunocytometry Systems, San Jose, CA) or FlowJo (Tree Star Inc, San Carlos, CA).

4.2.6. Culture of bone marrow derived macrophages and dendritic cells

Macrophages and dendritic cells were generated from bone marrow of C57BL/6, CD40^{-/-} MHC II^{-/-} or $\beta 2\text{m}^{-/-}$ mice, as described previously (146).

4.2.7. Macrophage killing assay and nitrite production

Bone marrow derived macrophages were harvested from C57BL/6 and CD40^{-/-} mice using standard procedure (146). After 6 days of culture macrophages were plated in U-bottom, 96-well plates (triplicates) at 2×10^5 /well. Macrophages were infected with *M. tuberculosis* at MOI 1.5 overnight.

The next day T cells were harvested from the lungs of 4-week infected mice, and single cell suspensions were obtained. Lung cells were divided into two samples. In unfractionated sample, macrophages were removed by incubating cell suspensions in NUNC LabTek plates at 37°C for 2 hours. In CD8-depleted sample, cells were incubated with 1:4 dilution of hybridoma 2.43 supernatant (anti-CD8) at 4°C for 30 minutes. Cells were washed once, and the cell pellet was suspended in T cell media (RPMI, 10% FBS, 1mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES, 50 μ M 2-ME (Sigma)). Cells were added to goat anti-rat IgG (Zymed Laboratories) coated plates (10 μ g/10ml of 0.05M Tris buffer/plate; 4°C; O/N) and incubated at room temperature for 1 hour. Following incubation, the non-adherent cells were removed, and plates were washed twice with PBS. Cells were counted and suspended at 1×10^6 /ml. Efficiency of CD8⁺ T cell depletion was confirmed by flow cytometry.

After overnight infection, macrophages were washed gently twice with warm DMEM and 200 μ l/well of T cell media, 250U/ml IFN- γ + 3 μ g/ml LPS, CD8-depleted lung cells (2×10^5 /well) or unfractionated lung cells (2×10^5 /well) were added to the wells. At this time point, macrophages were lysed in a subset of wells to determine the input number of intracellular bacteria.

Following 3-day incubation at 37°C, the number of intracellular bacteria was determined by lysing adherent macrophages with 1% saponin for 10 minutes. Cell lysates were collected,

sonicated for 10 seconds, and serial dilutions were plated on 7H10 agar plates. Nitrite in the supernatants was measured by Greiss assay (116).

4.2.8. Functional characterization of CD40^{-/-} and WT dendritic cells

Uninfected and *M. tuberculosis*-infected wild-type dendritic cells were treated with stimulating anti-CD40 antibody (BD Pharmingen; clone 3/23) or rIgG2a isotype control (BD Pharmingen; clone A110-2) at 3.5 µg/10⁶ cells/ml on ice for 30 minutes. Following antibody treatment uninfected and *M. tuberculosis*-infected wild-type and CD40^{-/-} dendritic cells were seeded at 1 x 10⁶/ml in 24-well plates in dendritic cell media supplemented with GM-CSF and IL-4 (1000 U/ml) for 24 hours. Supernatants were filter sterilized and the amount of IL-12 was determined by ELISA (see below). Dendritic cells were stained with the anti-mouse CD4, CD8, CD3, MHC Class I, MHC Class II and B7.2 antibodies. Flow cytometric analysis revealed that dendritic cell preparations used for either *M. tuberculosis* infection or HSP70 ligation experiments did not contain CD3⁺ T cells, and that they expressed high levels of MHC Class I, MHC Class II and B7.2.

For Hsp70 binding experiments, uninfected wild-type and CD40^{-/-} dendritic cells were incubated with either 10 µg/ml or 1 µg/ml of purified, LPS-free Hsp70, which was generously provided by Dr. John Belisle (the NIH Tuberculosis Reagents Contract NO1 AI-75320).

4.2.9. *In vitro* priming assay

Spleens were harvested from uninfected C57BL/6 mice, and single cell suspensions were obtained by crushing the organs through 70 µm cell strainers. Red blood cells were lysed with NH₄-Tris solution at room temperature for 2 minutes, followed by two washes in PBS.

Splenocytes were treated with anti-B220 antibody (BD Pharmingen; clone RA3-6B2) at $10\ \mu\text{g}/10^7$ cells/ml at 4°C for 30 minutes. After 30-minute incubation, unbound antibody was removed by washing cells once in PBS, and the cells were added to goat anti-rat IgG coated plates (Zymed Laboratories) for 1 hour at room temperature. At the end of 1-hour incubation, non-adherent cells were collected and plates were washed twice with PBS. Efficiency of B cell depletion was confirmed by flow cytometry. The remaining splenocytes were cultured in T cell media supplemented with 20 U/ml of IL-2 (Roche) with either *M. tuberculosis*-infected CD40^{-/-} or wild-type dendritic cells at 1:10 (DC:T cell) ratio for 7 days.

In IL-12 rescue assay, recombinant murine IL-12 (a generous gift from Genetics Institute, Cambridge, Massachusetts) was added to CD40^{-/-} DC–T cell co-cultures at 50ng/ml. Midway through T cell–DC co-culture, 100 μl of supernatants was removed from the wells and the amount of IL-12 was measured using ELISA. 100 μl of fresh T cell media was added supplemented with IL-2 (20 U/ml) for another 4 days of culture.

At the end of a 7-day incubation, cells were counted and used in an IFN- γ ELISPOT to determine the frequency of IFN- γ producing cells in response to T cell media (negative control), ConA (positive control; 10 μg /well (Sigma)), uninfected and *M. tuberculosis*-infected dendritic cells (1:2 DC:T cell ratio).

4.2.10. IL-12 immunotherapy in CD40^{-/-} and WT mice

CD40^{-/-} and WT mice were administered 300 ng of recombinant murine IL-12 (a generous gift from Immunex) three times a week. The treatment commenced on the first day of infection and continued for the next three weeks. At weekly time points lung and lymph nodes were harvested (week 1 – week 5), and analyzed for cell composition by flow cytometry and

IFN- γ production was evaluated by ELISPOT as described below. In addition, lung and lymph node tissues were snap frozen, and subsequently isolated RNA was used to determine IFN- γ mRNA expression by RT-PCR as described below.

4.2.11. ELISA

The amount of IL-12 was quantitated using ELISA as described previously (116). The capture anti-IL12 antibody (Biosource International; clone C15.6) was used at 4 μ g/ml in the binding buffer (0.1M Na₂HPO₄, pH 9.0) overnight at 4°C. The biotinylated anti-IL12 antibody (Biosource International; clone 17.15), which detects both p40/p70 heterodimer, was added at 2 μ g/ml in the incubation buffer for 1 hour at room temperature.

4.2.12. ELISPOT

Millipore Multiscreen 96-well MAIPS4510 plates (Millipore Corp, Bedford, MA) were coated with capture anti-IFN- γ antibody (BD Pharmingen; clone R4-6A2) in PBS at 10 μ g/ml overnight at 4°C.

The next day, the plates were washed with PBS, and blocked with RPMI/15% FBS for 1–2 hours at room temperature. Lung and lymph node single cell suspensions were prepared as described previously (146), and plated at 80,000 lung cells/well or 150,000 lymph node cells/well in T cell media supplemented with IL-2 at final concentration of 20 U/ml. Lung and lymph node cells were either cultured in T cell media alone, ConA (10 μ g/well (Sigma)), uninfected and *M. tuberculosis*-infected wild-type dendritic cells (total IFN- γ production), MHC II-/- dendritic cells (IFN- γ production by CD8⁺ T cells), and β 2m-/- dendritic cells (IFN- γ production by CD4⁺ T cells) at 1:2 (DC:T cell) ratio at 37°C for ~40 hours.

Following incubation plates were washed with PBS/0.1% Tween-20 and biotinylated anti-IFN- γ antibody (BD Pharmingen; clone XMG 1.2) was added at 5 μ g/ml in PBS/0.5%BSA/0.1% Tween-20 at 37°C for 2 hours. Avidin Peroxidase Complex (PK-6100; Vector Laboratories) was prepared as directed by the manufacturer and added to the plates at 100 μ l/well for 1 hour at room temperature. Following incubation, plates were washed with PBS/0.1% Tween-20, and developed by adding Vectastain AEC substrate (SK-4200; Vector Laboratories) prepared according to manufacturer's instructions. The spot forming units (SFU) per well were counted using ELISpot reader (Cellular Technology Ltd, Cleveland, OH). The cut off number of SFU accurately measured by the ELISpot reader is 1500 SFU/well. In IL-12 rescue assay, the wells that were "too numerous to count" (TNTC) or were solid red were estimated to be >1500 SFU/well.

4.2.13. Quantitative RT-PCR

Total lung and lymph node RNA was extracted using Trizol (Life Technology, Green Island NY) and RNA extraction kit as directed by the manufacturer (Qiagen; Valencia, CA). cDNA synthesis was performed using Superscript II enzyme system according to the manufacturer's instructions (Quiagen). We adopted a relative gene expression method as described previously (213). In our assay, we used RNA isolated from the lungs of uninfected mice as a calibrator and we used HPRT as a normalizer gene. Relative gene expression was calculated as $2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t$ (gene of interest) – C_t (normalizer) and the $\Delta\Delta C_t = \Delta C_t$ (sample) - ΔC_t (calibrator). We used published sequences for the IL-12 primer and probe sets (214) at 400 nM and 250 nM concentrations, respectively.

4.2.14. RNase Protection Assay

A custom-made template set mck-2b (NOS2, IL-4, IL-12p40, TNF- α , IL-1 β , IL-1 α , IFN- γ), template set mck-1 (IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, IFN- γ) and multiprobe RNase protection assay (RPA) system (BD Pharmingen) were used to determine mRNA levels for genes of interest at designated time points. The relative gene expression was quantified by densitometer (ImageQuant Software; Molecular Dynamics, Sunnyvale, CA) and compared to the abundance of the housekeeping gene, L32.

4.2.15. Statistics

The results represent the mean \pm standard error of mean. Statistical significance was calculated using student t-test. For comparison of CFU between CD40^{-/-} and wild-type mice, the raw data were transformed into log numbers prior to statistical analysis. * p-value \leq 0.05; ** p-value \leq 0.01.

4.3. Results

4.3.1. CD40^{-/-} mice are susceptible to aerosol *M. tuberculosis* infection

The course of infection was compared in CD40^{-/-} and wild-type mice after low dose aerosol infection with virulent *M. tuberculosis* (20–50 CFU/mouse). Bacterial numbers were similar for the first 2 weeks post-infection between the two groups of mice. As colony forming units (CFU) began to plateau in the wild-type mice at 3 weeks post-infection, bacterial numbers continued to rise in the lungs and spleen of the CD40^{-/-} mice reaching 500-1000 fold higher bacterial burden by 4 weeks post-infection (Figure 4A and 4B). Indeed, 40% of the CD40^{-/-} mice were moribund between 3 and 4 weeks post-infection. CD40^{-/-} mice that survived this crisis point had reduced bacterial numbers by 5 weeks post-infection (Figure 4A and 4B). A small number of surviving CD40^{-/-} mice were followed up to 5 months post-infection; these mice had excessive lung pathology and 10-1000 fold higher CFU in the lungs compared to wild-type mice.

Histological analysis did not reveal obvious differences in the infiltration of cells into the lungs in the first three weeks of infection (Figure 4C). Early in infection, CD40^{-/-} mice formed granulomas comparable in numbers to those found in the wild-type mice. Analysis of lung sections from CD40^{-/-} mice revealed the presence of unusual, multi-nucleated giant cells at 3 weeks post-infection (Figure 4C). These giant cells are the hallmark of human tuberculosis, but are rarely found in mice except in chronic infection as a result of long-term inflammation (data not shown). The most dramatic difference between the lungs of CD40^{-/-} and wild-type mice was detected during the crisis point at 4 weeks post-infection (Figure 4C). There was massive necrosis in the lungs of CD40^{-/-} mice resulting in extensive consolidation of airspaces and death of mice.

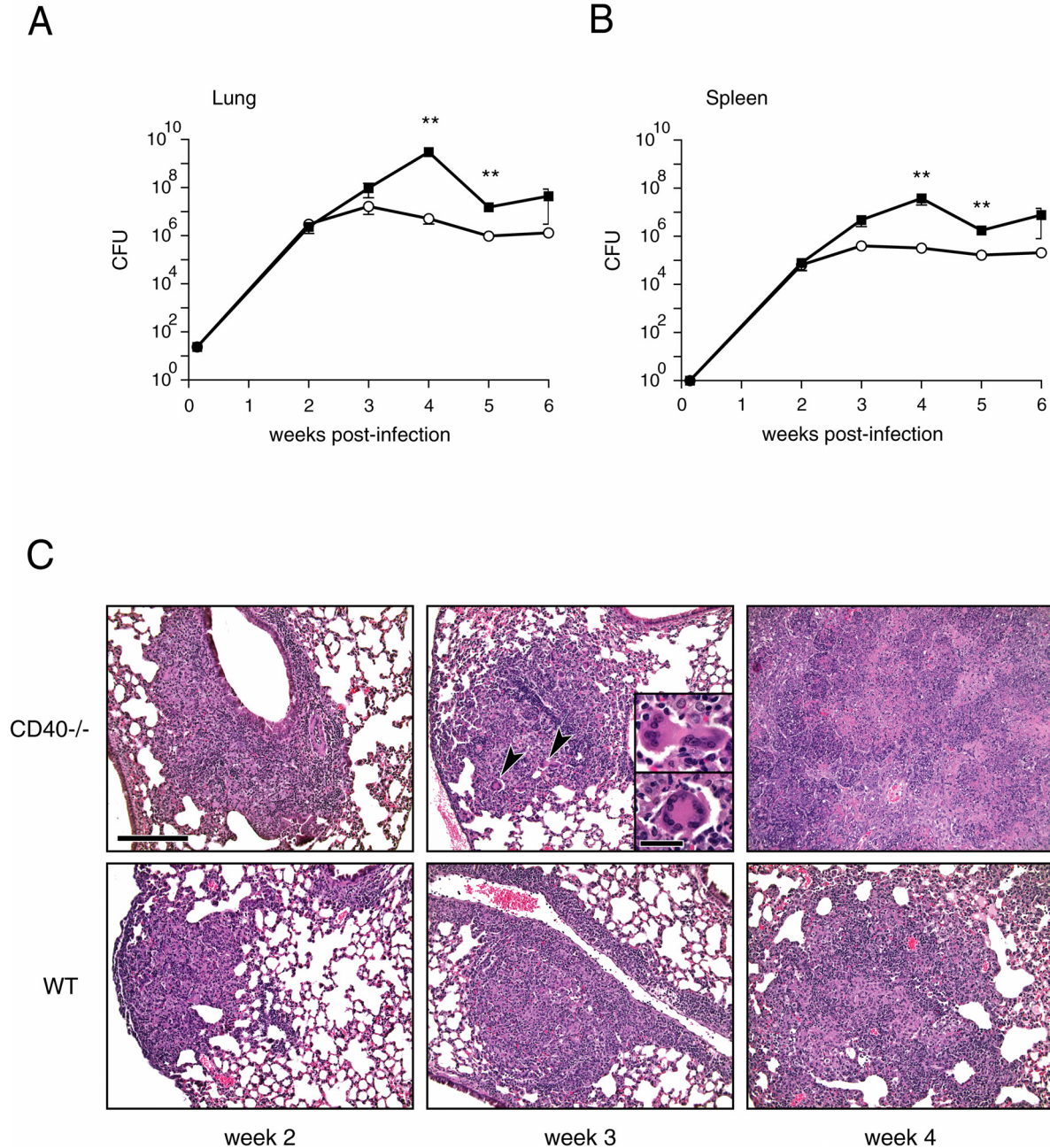


Figure 4 CD40^{-/-} mice are susceptible to aerosol *M. tuberculosis* infection

(A and B) CD40^{-/-} (■) and C57BL/6 (○) mice were infected with ~20-50 CFU of *M. tuberculosis* via aerosol. At weekly time points the number of CFU was determined by plating serial dilutions of the lung (A) and spleen (B) homogenates.

(C) At 2, 3 and 4 weeks post-infection lung sections of CD40^{-/-} and WT mice were stained with hematoxylin and eosin. Scale bar equals 200 μm, inset scale bar is 30 μm. The data are representative of three experiments, with 4 mice per experimental group at each time point.

4.3.2. CD40 ligation is not required for the induction of mycobactericidal mechanisms in macrophages

Production of reactive nitrogen intermediates (RNI), such as NO, via the inducible nitric oxide synthase (NOS2) pathway by activated macrophages is an important component of macrophage-mediated defense against *M. tuberculosis* (105, 215). CD40/CD40L interaction was critical for T cell dependent activation of macrophages resulting in RNI production and microbicidal activity against *Leishmania* and other intracellular pathogens (202-205). To address the role of CD40/CD40L interaction in macrophage activation, *in vivo* expression of CD40 following *M. tuberculosis* infection was examined. By 1 week post-infection, only 5% of lung cells within the R1 gate expressed CD40, but this number increased to 50% by 4 weeks post-infection (Figure 5A).

To test the dependence of macrophage effector functions on CD40 ligation, the ability of CD40^{-/-} and wild-type macrophages to limit the growth of intracellular mycobacteria and produce nitrite (a measure of RNI) was evaluated *in vitro*. Bone marrow derived CD40^{-/-} and wild-type macrophages were infected with *M. tuberculosis* (Figure 5B, input) and cultured for three days in T cell media alone, IFN- γ /LPS, or with unfractionated or CD8-depleted lung T cells isolated from 4-week infected wild-type mice. CD40^{-/-} and wild-type macrophages were equally efficient at reducing the numbers of intracellular mycobacteria after IFN- γ /LPS or T cell activation (Figure 5B). Furthermore, similar amounts of nitrite were produced when infected CD40^{-/-} and wild-type macrophages were activated with IFN- γ /LPS or mycobacteria-specific T cells (Figure 5C). No reduction in the number of intracellular mycobacteria or nitrite production was observed when macrophages were cultured with naïve splenic T cells (data not shown).

To analyze *in vivo* macrophage activation during infection, lung sections were stained for the expression of NOS2. There was no apparent difference in the amount or the pattern of NOS2 staining within the granulomas of CD40^{-/-} and wild-type mice (Figure 5D). Collectively these results indicate that CD40^{-/-} macrophages did not differ significantly from wild-type macrophages in their intrinsic ability to produce RNI or limit the growth of intracellular bacteria. Hence, CD40/CD40L interaction is not pivotal to the induction of NOS2 expression in *M. tuberculosis*-infected mice.

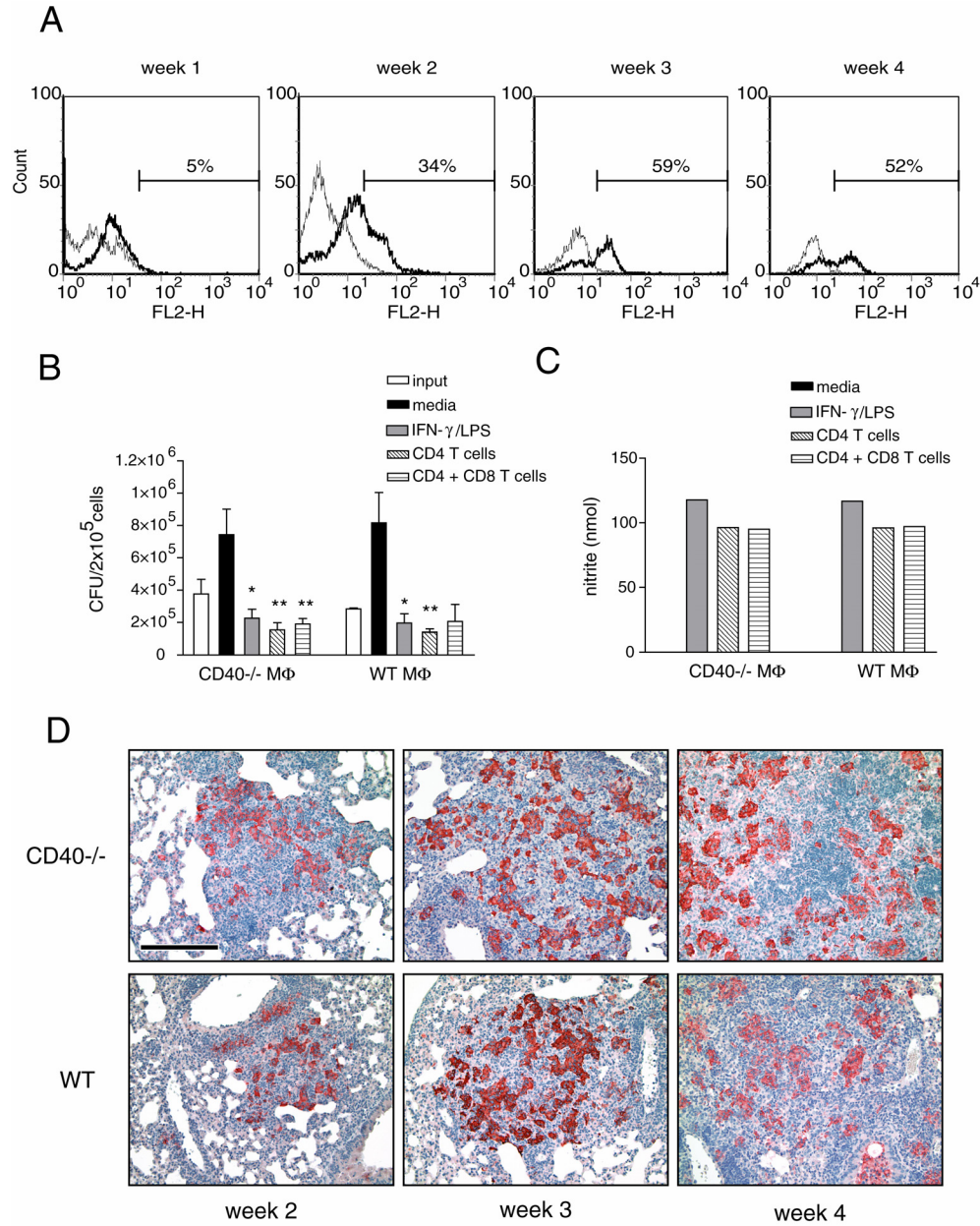


Figure 5 CD40 ligation is not required for the induction of mycobactericidal state in macrophages

(A) Expression of CD40 on lung cells during the course of aerosol *M. tuberculosis* infection was determined by flow cytometry. Lung cells from wild-type mice were stained with anti-CD40 antibody (thick line) or isotype control (thin line) and the percentage of CD40 positive cells within R1 gate was determined.

(B) The ability of CD40^{-/-} and wild-type MΦ to reduce the number of intracellular bacteria was assessed by an *in vitro* macrophage killing assay as described in Materials and Methods. Error bars are standard error of mean and the p values were calculated by comparing each condition to the input CFU. * p-value ≤ 0.05; ** p-value ≤ 0.01.

(C) The amount of nitrite in supernatants of resting MΦ (media alone) or IFN-γ/LPS and T cell activated MΦ was determined by a Greiss assay. The data are representative of three experiments (B-C) with 4 mice per experimental group at each time point.

(D) Expression of iNOS in the lung sections of CD40^{-/-} and WT mice.

4.3.3. CD40^{-/-} dendritic cells are inefficient in priming naïve T cells despite normal maturation and migration into the lung draining lymph nodes

CD40/CD40L interaction has an important role in the maturation and activation of APCs, characterized by up-regulation of co-stimulatory and antigen-presenting molecules and production of inflammatory cytokines (201, 216). To ascertain the role of CD40/CD40L interaction in dendritic cell activation, cell surface molecule expression as a result of *M. tuberculosis* infection and CD40 ligation was examined. Infection of both wild-type and CD40^{-/-} dendritic cells with *M. tuberculosis* resulted in up-regulation of MHC Class I, MHC Class II and B7.2 (data not shown). Ligation of CD40 on infected dendritic cells with stimulating anti-CD40 antibody did not significantly increase expression of co-stimulatory and antigen-presenting molecules suggesting that *M. tuberculosis* infection alone was sufficient to mature CD40^{-/-} and wild-type dendritic cells (data not shown). *In vivo*, migration of dendritic cells into the lung draining lymph nodes of wild-type and CD40^{-/-} mice after aerosol challenge was comparable (Figure 6A). These results indicate that CD40^{-/-} and wild-type dendritic cells are equally equipped with antigen presenting and co-stimulatory machinery and exhibit no defect in their *in vivo* migratory abilities.

Next, we investigated the importance of CD40 ligation in cytokine production by dendritic cells and their ability to prime *M. tuberculosis*-specific T cell responses *in vitro*. The most striking difference between wild-type and CD40^{-/-} dendritic cells was observed in their capacity to produce IL-12 following *M. tuberculosis* infection. Incubation with stimulating anti-CD40 antibody or infection with *M. tuberculosis* in the absence of T cells induced a 4-fold increase in IL-12 production by wild-type dendritic cells (Figure 6B). In contrast, *M. tuberculosis* infection of CD40^{-/-} dendritic cells failed to induce IL-12 production above the

baseline. Since there is a strong correlation between the IL-12 production and generation of a strong Th1 response, we next evaluated the ability of CD40^{-/-} and WT dendritic cells to prime naïve splenocytes. Splenocytes from naïve wild-type mice were incubated with *M. tuberculosis*-infected wild-type or CD40^{-/-} dendritic cells. Following 7-day *in vitro* priming, T cells were used in ELISPOT assays. Priming with wild-type or CD40^{-/-} dendritic cells resulted in preferential expansion of CD8⁺ T cells, with < 2% residual CD4⁺ T cells (data not shown). Therefore, the number of spot forming units (SFU) in the ELISPOT assays after *in vitro* priming is mainly attributed to IFN- γ production by CD8⁺ T cells. The frequency of *M. tuberculosis*-specific IFN- γ producing T cells after priming with infected wild-type dendritic cells was 2-fold higher than in T cell cultures that were primed with infected CD40^{-/-} dendritic cells (Figure 6C).

As expected the level of IL-12 in the CD40^{-/-} DC–T cell cultures was also 4-fold lower than in the wild-type DC–T cell co-cultures from the *in vitro* priming experiments (Figure 6D). Consequently, the priming of IFN- γ T cell responses by CD40^{-/-} dendritic cells was inefficient due to low production of IL-12 following *M. tuberculosis* infection. These findings suggest that CD40 ligation on dendritic cells is essential for optimal production of IL-12 and priming of *M. tuberculosis*-specific IFN- γ T cell responses *in vitro*.

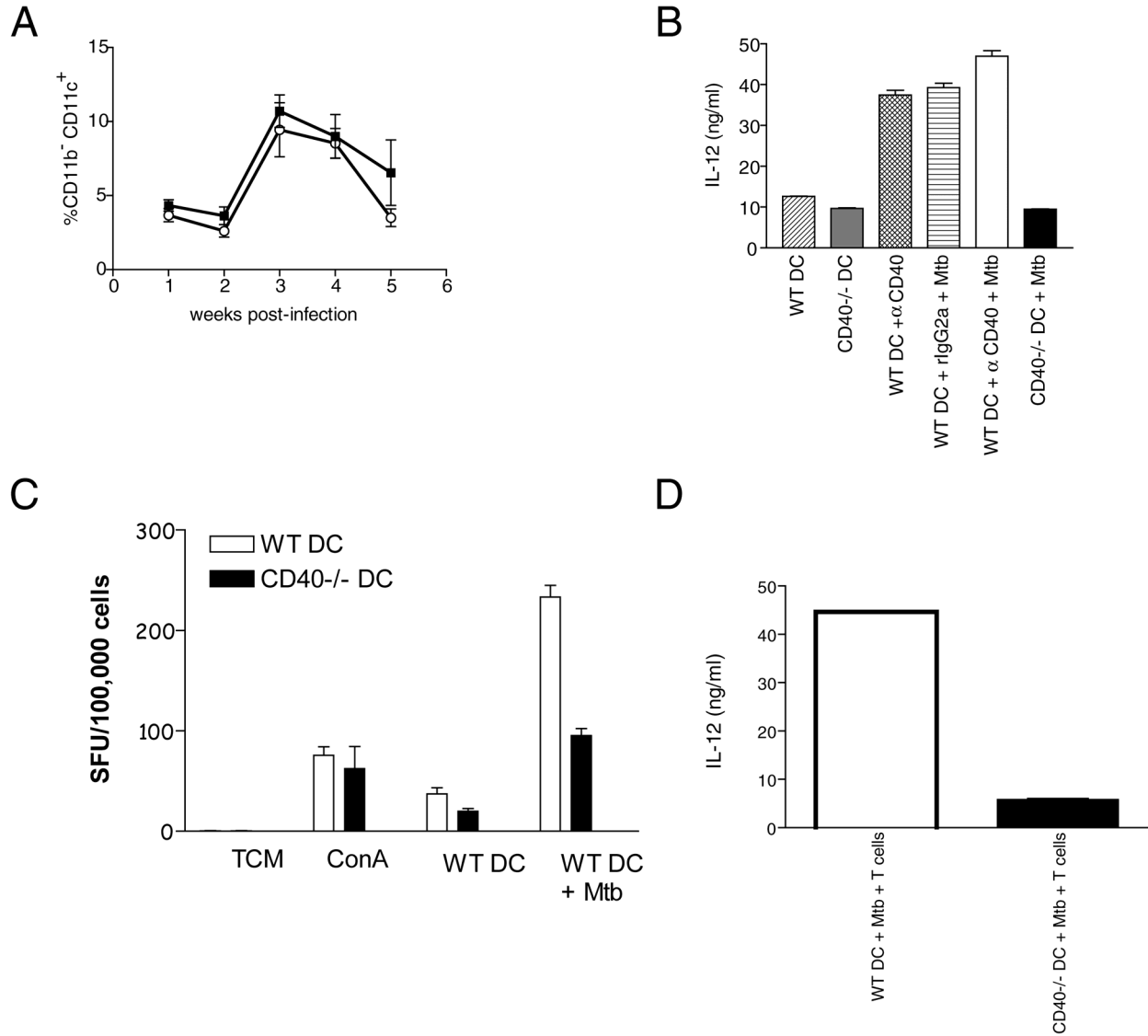


Figure 6 CD40^{-/-} DCs are inefficient at priming naïve T cells and show diminished IL-12 production *in vitro*

(A) Single cell suspensions from lymph nodes of CD40^{-/-} (●) and wild-type (○) mice were stained with anti-CD11c antibodies. The percentage of CD11c⁺ cells within R1 gate is shown.

(B) The amount of IL-12 produced by uninfected or *M. tuberculosis*-infected CD40^{-/-} dendritic cells and uninfected and *M. tuberculosis*-infected wild-type dendritic cells treated with either stimulating anti-CD40 antibody or isotype control was determined by ELISA.

(C) The ability of CD40^{-/-} and wild-type dendritic cells to prime naïve T cells was assessed by *in vitro* priming assay as described in Materials and Methods.

(D) IL-12 levels in WT dendritic cells - T cell or CD40^{-/-} dendritic cells - T cell co-cultures during the *in vitro* priming experiment were quantified by ELISA. The data are representative of three (B and C) or two (D) experiments.

4.3.4. Susceptibility of CD40^{-/-} mice to *M. tuberculosis* is associated with impaired IFN- γ production *in vivo*

Given that CD40^{-/-} dendritic cells were inefficient at priming naïve T cells *in vitro*, the requirement for CD40 in induction of protective, *M. tuberculosis*-specific Th1 T cell responses *in vivo* was investigated. To establish the existence of IL-12 deficiency in CD40^{-/-} mice *in vivo*, we measured the amount of IL-12 mRNA in the lungs and lymph nodes of CD40^{-/-} and WT mice using quantitative RT-PCR. Our results indicate that there was substantially less IL-12 in the lymph nodes of CD40^{-/-} mice at 2 and 3 weeks post-infection during the peak of priming in wild-type mice (Figure 7A). In addition, there was a significant difference in the levels of IL-12 in the lungs of CD40^{-/-} and WT mice throughout the acute infection (Figure 7B).

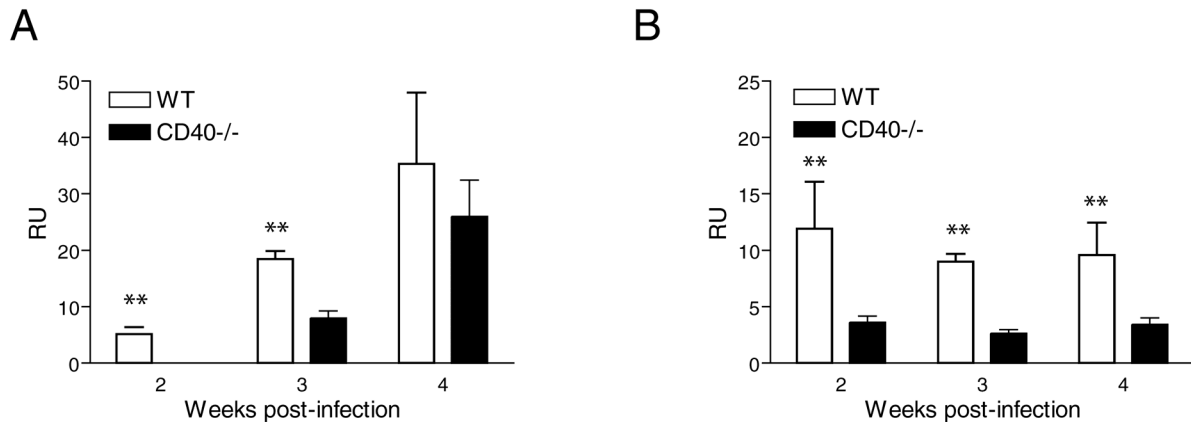


Figure 7 Diminished IL-12 production in the lymph nodes and the lungs of CD40^{-/-} mice

(A and B) Expression of IL-12 mRNA in the lymph nodes (A) and lungs (B) of CD40^{-/-} and wild-type mice as measured by quantitative RT-PCR. The data show significant reduction in IL-12 mRNA expression in CD40^{-/-} mice.

CD40^{-/-} mice had substantially fewer CD4⁺ and CD8⁺ T cells in the lungs than wild-type mice (Figure 8A). The most striking difference was seen at 4 weeks following aerosol challenge when many CD40^{-/-} mice succumbed to infection. This is the time of the peak response in wild-type mice, after which the T cell numbers contract as the infection is brought under control. CD40^{-/-} mice that survived the crisis point at 4 weeks post-infection had wild-type numbers of T cells at 5 weeks post-infection.

The frequency of IFN- γ producing T cells in the lymph nodes and the lungs of infected CD40^{-/-} and wild-type mice following *M. tuberculosis* infection was determined by ELISPOT assay. T cells isolated from the lymph nodes and lungs of infected mice were incubated with uninfected and infected wild-type dendritic cells (to measure total IFN- γ secreting T cells) or uninfected and infected β 2m^{-/-} dendritic cells (to measure IFN- γ producing CD4⁺ T cells) (Figure 8B). In wild-type mice, IFN- γ producing T cells were detected in the lymph nodes and lungs as early as 2 weeks post-infection (Figure 8B and 8C). The numbers of IFN- γ producing T cells increased in the lungs of wild-type mice up to 4 weeks post-infection, when the bacterial numbers stabilized (Figure 8C). In contrast, CD40^{-/-} mice suffered from a major defect in the priming of IFN- γ secreting T cells in the lymph nodes, which was most obvious at the peak priming point of 2 weeks post-infection (Figure 8B and 8C). This deficit in priming resulted in overall weaker IFN- γ responses in the lungs of CD40^{-/-} mice, which finally reached wild-type levels at 5 weeks post-infection. Both CD4⁺ and CD8⁺ T cell responses were impaired; however the absence of CD40 molecule had a greater impact on CD4⁺ T cells (Figure 8D). These results were confirmed by IFN- γ intracellular cytokine staining of T cells from the lungs of wild-type and CD40^{-/-} mice at each time point (data not shown).

M. tuberculosis-infected CD40^{-/-} and wild-type dendritic cells were equally capable of inducing T cells from infected mice to produce IFN- γ upon *in vitro* re-stimulation (data not shown). Furthermore, intracellular cytokine staining revealed similar percentages of IFN- γ positive cells within CD4⁺ and CD8⁺ gates after stimulation with anti-CD3/CD28 antibodies, indicating that T cells that had infiltrated the lungs of CD40^{-/-} mice function independently of CD40/CD40L interaction (data not shown). These results suggest that CD40 is critical for the initiation of T cell responses within the secondary lymphoid organ, but not at the effector stage of T cell function within the lung following *M. tuberculosis* infection.

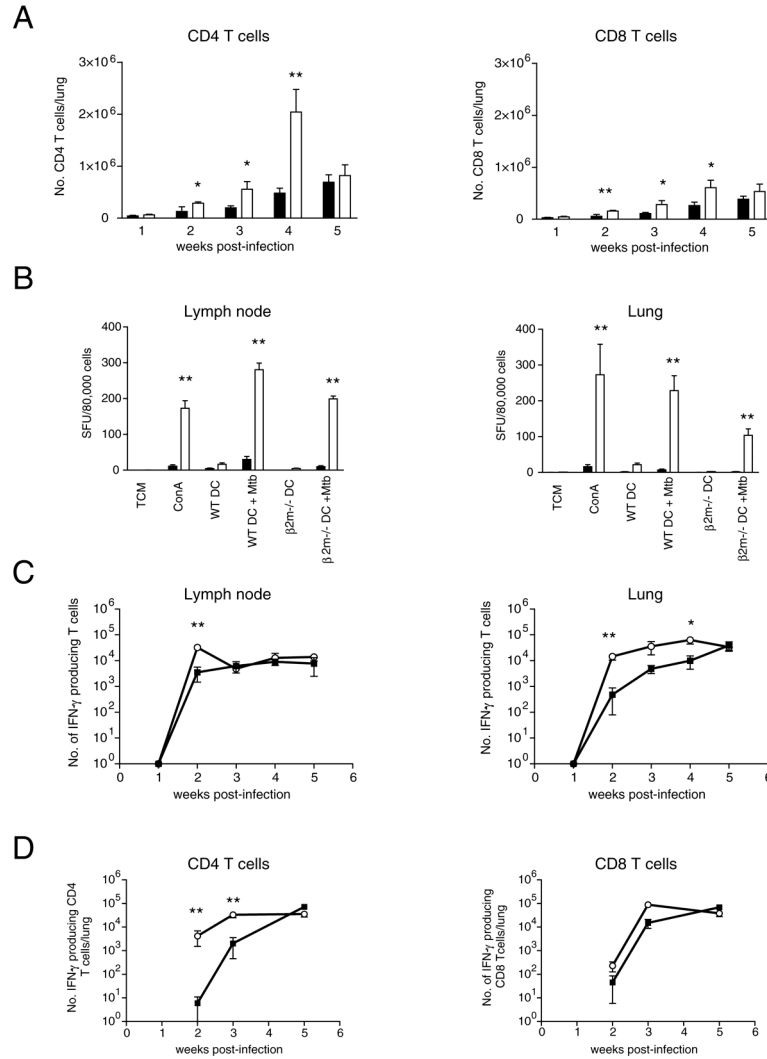


Figure 8 CD40^{-/-} mice show delayed priming of IFN- γ producing T cells in the lymph nodes and overall weaker IFN- γ responses at the site of infection

(A) Lung cells from CD40^{-/-} (■) and wild-type (□) mice were stained with anti-CD4 and anti-CD8 antibodies. The data are representative of 3 experiments, and 5-6 mice per experimental group were used at each time point.

(B) The number of IFN- γ producing T cells from the lymph nodes and lungs of 2 week-infected CD40^{-/-} (■) and wild-type (□) mice was assessed by ELISPOT.

(C) The number of SFU per 150,000 of lymph node cells or 80,000 lung cells was used to calculate the frequency of IFN- γ producing cells in response to *M. tuberculosis*-infected wild-type dendritic cells. The number of total IFN- γ secreting T cells per lymph node or lung was calculated using the following formula: Number of IFN- γ producing cells = frequency x total number of cells per lymph node or lung. In each case, the number of SFU in response to uninfected dendritic cells was subtracted from the number of SFU in response to *M. tuberculosis*-infected dendritic cells before the calculations were made. (■) represent CD40^{-/-} and (○) represent wild-type mice.

(D) 80,000 lung cells from CD40^{-/-} (■) and wild-type (○) mice were incubated with either uninfected and *M. tuberculosis*-infected $\beta 2m^{-/-}$ dendritic cells or uninfected and *M. tuberculosis*-infected MHC II^{-/-} dendritic cells to estimate the number of IFN- γ producing CD4⁺ and CD8⁺ T cells, respectively.

4.3.5. Exogenous administration of IL-12 *in vitro* and *in vivo* improved the priming efficiency of CD40^{-/-} dendritic cells

The most striking difference between CD40^{-/-} and WT dendritic cells was in their ability to produce IL-12 upon *M. tuberculosis* infection. Diminished IL-12 production by infected CD40^{-/-} dendritic cells is the main cause for the poor priming of Th1 response, thus leading to the susceptibility of CD40^{-/-} mice to *M. tuberculosis*. We postulated that exogenous administration of IL-12 to *in vitro* CD40^{-/-} - T cell co-cultures and to *M. tuberculosis* infected CD40^{-/-} mice will lead to improved priming of IFN- γ producing T cells.

Exogenous administration of 50 ng/ml of recombinant murine IL-12 to CD40^{-/-} DC – T cell co-cultures significantly improved the priming capacity of CD40^{-/-} dendritic cells and resulted in the priming of high frequency of IFN- γ producing T cells (Figure 9).

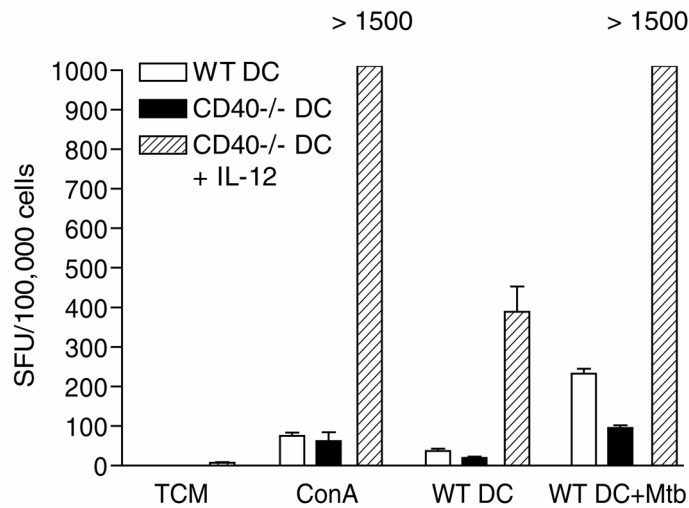


Figure 9 Exogenous administration of IL-12 improves the priming efficiency of CD40^{-/-} dendritic cells *in vitro*

Naïve splenocytes were stimulated with *M. tuberculosis*-infected WT dendritic cells, *M. tuberculosis*-infected CD40^{-/-} dendritic cells, and *M. tuberculosis*-infected CD40^{-/-} dendritic cells supplemented with 50 ng/ml of IL-12 for 7 days in *in vitro* priming experiment. Following seven days of incubation, splenocytes were collected, plated at 100,000 cells/well and the frequency of IFN- γ producing T cells in response to media, ConA, uninfected and *M. tuberculosis*-infected dendritic cells was estimated by ELISPOT.

IL-12 immunotherapy of 300 ng/mouse, three times a week for the first three weeks of infection, led to enhanced IFN- γ responses in CD40^{-/-} mice both at the RNA and protein level (Figure 10A – 10C). IL-12 treatment of both CD40^{-/-} and WT mice gave rise to the induction of a stronger Th1 response as evidenced by increased levels of IFN- γ mRNA and high frequency of IFN- γ producing T cells in the lymph nodes of treated mice (Figure 10A - 10B). Although enhanced expression of IFN- γ mRNA was observed in the lungs of WT mice, the frequency of IFN- γ producing T cells was reduced (Figure 10A – 10C). This enhanced expression of IFN- γ mRNA could be attributed to NK cells.

IL-12 immunotherapy resulted in the improved survival of CD40^{-/-} mice. All PBS treated mice had extensive lung pathology at 5 weeks post-infection (data not shown) and succumbed to infection by 2 months post-infection. None of the IL-12 treated mice showed signs of the disease and survived the challenge with the low dose of *M. tuberculosis* infection. The number of CFUs in the lungs and spleen of IL-12 treated CD40^{-/-} mice were not significantly different from wild-type mice, while PBS treated mice had a 100 fold higher bacterial burden at 5 weeks post-infection than IL-12 treated CD40^{-/-} and wild-type mice (Figure 11).

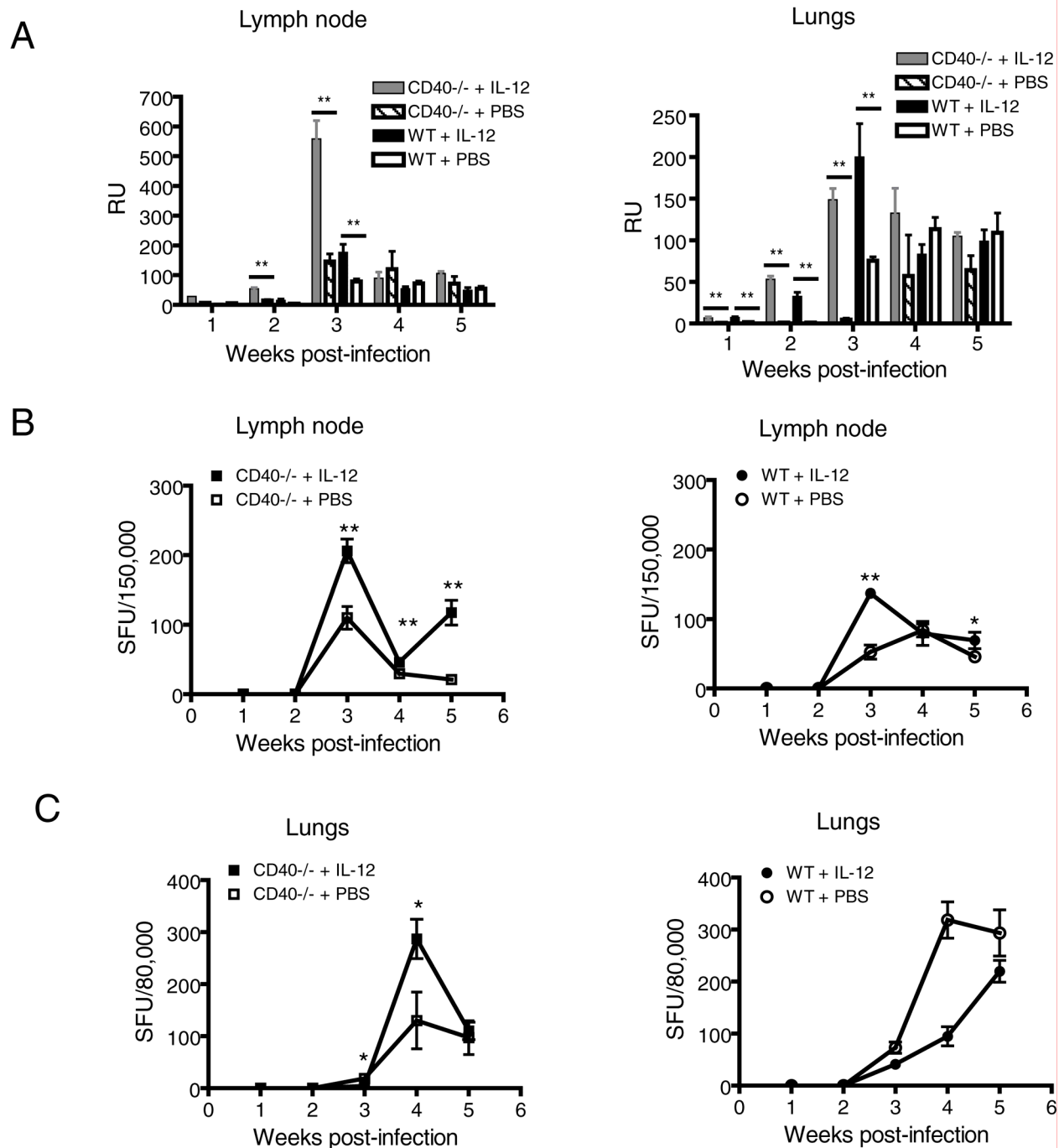


Figure 10 Exogenous administration of IL-12 to CD40^{-/-} mice enhanced the priming of IFN- γ response at mRNA and protein level

(A) RNA was isolated from the lymph nodes and lungs of IL-12 and PBS treated CD40^{-/-} and WT mice. The level of IFN- γ mRNA expression was determined by quantitative RT-PCR using relative expression method.

(B and C) The frequency of IFN- γ producing T cells in the lymph nodes (B) and the lungs (C) of IL-12 and PBS treated CD40^{-/-} and WT mice was determined by ELISPOT as described in the Materials and Methods.

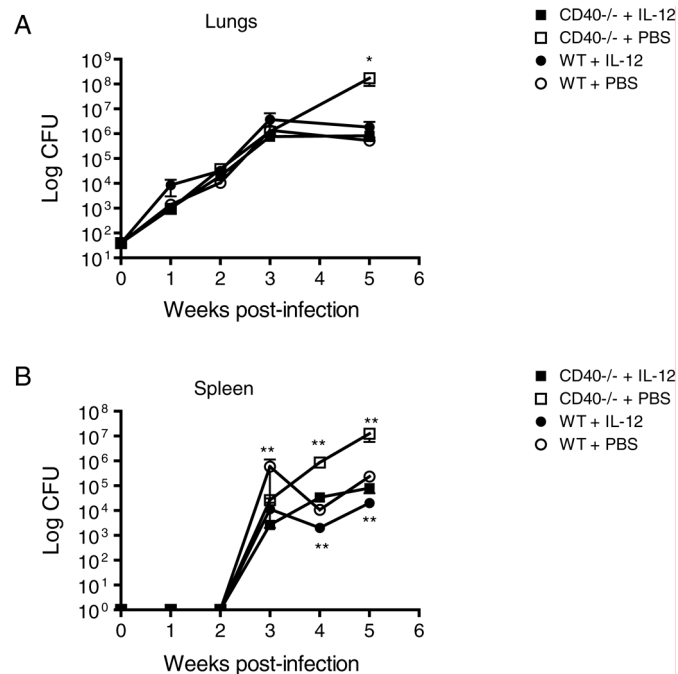


Figure 11 Exogenous administration of IL-12 to CD40^{-/-} mice resulted in better control of *M. tuberculosis* infection

(A and B) Colony forming units from the lungs (A) and the spleens (B) of IL-12 and PBS treated CD40^{-/-} and WT mice was determined by plating serial dilutions of lung and spleen homogenates on 7H10 plates. Top asterisks designate statistically significant differences between IL-12 and PBS treated CD40^{-/-} mice, while bottom asterisks denote significant differences between IL-12 and PBS treated wild-type mice.

4.3.6. CD40^{-/-} mice that survive the crisis point succeed in inducing IFN- γ responses

The finding that some CD40^{-/-} mice survived the crisis point, and could live up to 5 months post-infection albeit with a higher bacterial burden was intriguing. To investigate the differences between resistant and susceptible CD40^{-/-} mice, the cytokine profile in the lungs of CD40^{-/-} and wild-type mice was analyzed by RNase protection assay (RPA). At 4 weeks post-infection, during the crisis point, we extracted RNA from the lungs of four moribund CD40^{-/-} mice (Figure 12A; lanes 2, 4, 14, 16), one CD40^{-/-} mouse that was controlling infection (Figure

12A; lane 6), and three wild-type mice (Figure 12A; lanes 8, 10, 12). CD40^{-/-} mice that failed to control *M. tuberculosis* infection had ~1000-fold higher CFU in the lungs compared to wild-type mice. These mice had higher levels of IL-10 and IL-6 and no detectable IL-15 and IFN- γ mRNA (Figure 12A; lanes 2, 4, 14, 16). In contrast, the relatively healthy CD40^{-/-} mouse had ~10-fold more CFU in the lungs compared to wild-type mice, no IL-10 mRNA expression and normal levels of IFN- γ and IL-15 (Figure 12A, compare lane 6 to lanes 8, 10, 12). In the healthy CD40^{-/-} mouse, a high level of IL-6 mRNA was also detected indicating that irrespective of the disease status, the inflammation in the lungs of CD40^{-/-} mice was significant (Figure 12A, compare lane 6 to lanes 8, 10, 12).

As a group, CD40^{-/-} mice had significantly reduced levels of IFN- γ mRNA over the course of infection (Figure 12B) and higher levels of IL-10 at 4 weeks post-infection (Figure 12C). The increased level of IL-10 mRNA appears to be due to IL-10 production by macrophages as no IL-10 was detected within the lymphocyte population by intracellular cytokine staining or ELISPOT following specific and non-specific stimulation (data not shown). Expression of IL-10 and IL-6 is likely due to the inflammation and extensive necrosis in the lungs of CD40^{-/-} mice at 3 and 4 weeks post-infection. At 5 weeks post-infection no IL-10 mRNA was detected in the lungs of CD40^{-/-} and wild-type mice. CD40^{-/-} mice also had significantly less IL-12 mRNA in the lungs throughout the infection as shown by quantitative real-time RT-PCR (data not shown). No IL-4 mRNA was detected (data not shown).

These results confirmed that the susceptibility of CD40^{-/-} mice to progressive *M. tuberculosis* infection was associated with a defect in the ability of these mice to produce IFN- γ in the lungs during acute infection. Those mice that survived were eventually able to recruit IFN- γ producing T cells to the lungs.

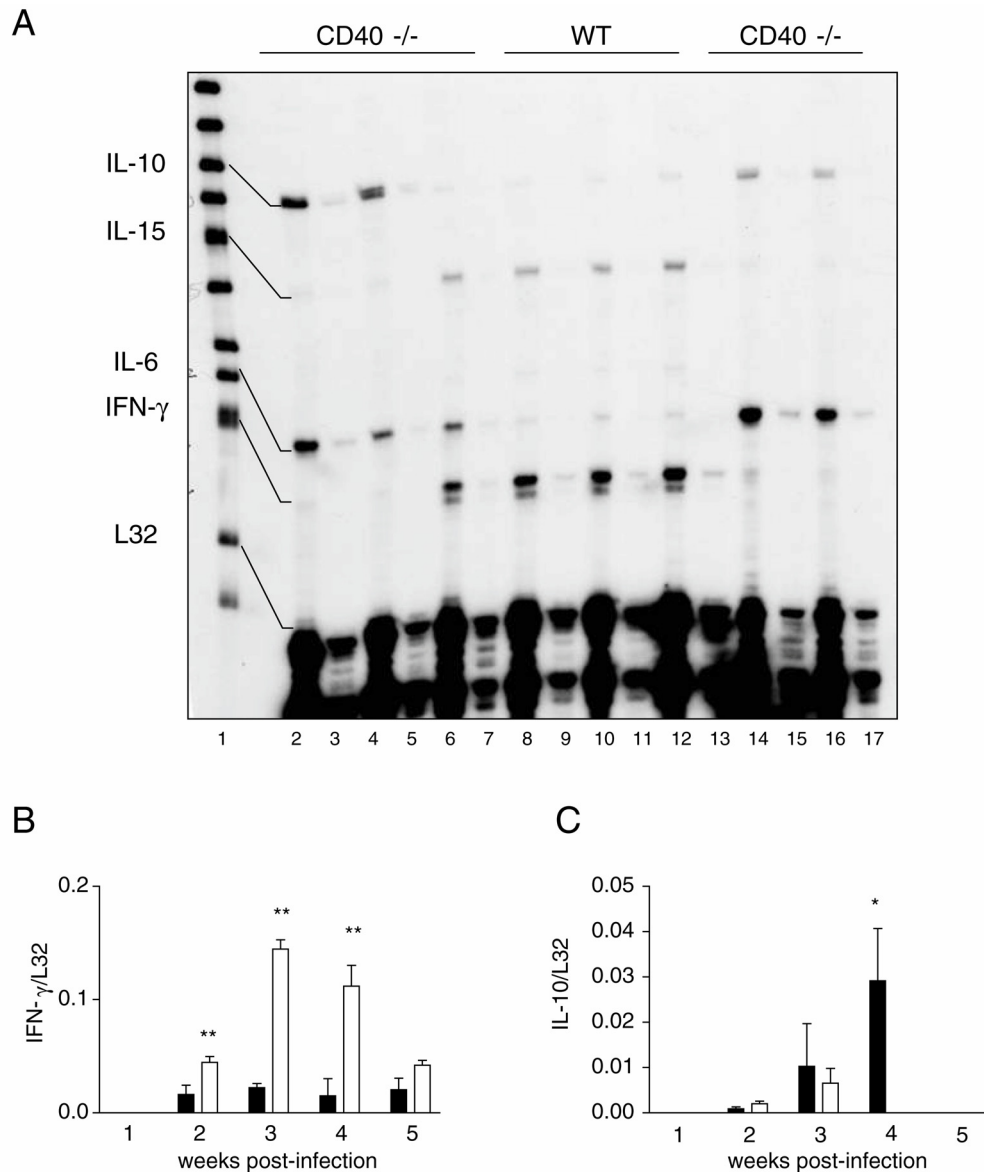


Figure 12 CD40^{-/-} mice that controlled *M. tuberculosis* infection succeeded in recruiting IFN- γ producing T cells

(A) At 4 weeks post-infection RNA was extracted from the lungs of four moribund CD40^{-/-} mice (lanes 2, 4, 14, 16), one CD40^{-/-} mouse that controlled infection (lane 6) and three WT mice (lanes 8, 10, 12) and analyzed by RPA. Lane 1 contains unprotected probe (marker) and each lane represents protected probe from an individual mouse or its 1:10 dilution.

(B-C) mRNA levels for IFN- γ and IL-10 were determined by RPA at weekly time points from the lungs of infected CD40^{-/-} (■) and wild-type (□) mice. The results represent the mean ratio of the gene of interest and L32 house keeping gene for 4 mice per experimental group per time point. The data are representative of two experiments.

4.3.7. Infection of CD40^{-/-} mice with higher inoculum rescued CD40^{-/-} mice from death

The duration and strength of TCR-mediated signals may dictate the level of co-stimulation required to achieve T cell activation (217, 218). Therefore, the antigen dose or number of priming events may determine the requirement for CD40/CD40L interaction in the induction of *M. tuberculosis*-specific T cell responses. To address this question, CD40^{-/-} mice were infected with 2×10^5 CFU/mouse intravenously, a sublethal dose in wild-type mice. CD40^{-/-} mice had approximately 10-fold higher number of CFU in the lungs 3 weeks after intravenous infection with *M. tuberculosis* (Figure 13A). In contrast to aerosol infected CD40^{-/-} mice in which the number of CFU continued to rise to high levels, CD40^{-/-} mice maintained slightly higher but stable bacterial numbers after intravenous infection (Figure 13A). None of CD40^{-/-} mice died after intravenous *M. tuberculosis* infection during the study. Except for the 2-week time point, the overall numbers of CD4⁺ and CD8⁺ T cells in the lungs of CD40^{-/-} mice were comparable to those in wild-type mice (Figure 13B). Normal priming in the lymph nodes and only slightly lower IFN- γ responses were observed in the lungs of CD40^{-/-} mice at 2 weeks post-infection (Figure 13C). No difference in the number of IFN- γ producing T cells was detected in the spleens of CD40^{-/-} and wild-type mice following intravenous infection (Figure 13C, spleen). After aerosol challenge, there were on average 500 and 5000 IFN- γ producing T cells in the lungs of CD40^{-/-} mice at 2 and 3 weeks, respectively (Figure 13C, lung). After higher antigenic challenge a 17-fold and 2-fold increase in the number of IFN- γ producing T cells was detected in the lungs of CD40^{-/-} mice at 2 and 3 weeks, respectively (Figure 13C, lung).

Aerosol infection with a higher inoculum (2-3 fold higher, ~100 CFU) was performed to address whether higher antigen load or a systemic infection increased resistance of CD40^{-/-} mice

to *M. tuberculosis*. Similarly to intravenous infection, none of CD40^{-/-} mice died after high dose aerosol infection. CD40^{-/-} mice had slightly higher, but nevertheless controlled, bacterial loads in the lungs and spleen after high dose aerosol infection (data not shown).

When compared to WT mice, CD40^{-/-} mice infected with a higher inoculum via aerosol had fewer CD4⁺ and CD8⁺ T cells in the lungs at 3 weeks post-infection; however, this was compensated for by a massive expansion of CD4⁺ and CD8⁺ T cell responses at 4 weeks post-infection (data not shown). Induction of IFN- γ T cell responses was robust after higher dose *M. tuberculosis* infection (data not shown). When compared to low dose infection, the number of IFN- γ producing T cells was 7-fold higher in the lymph nodes of CD40^{-/-} mice at 3 weeks after high dose infection, leading to 8.6-fold and 8-fold higher numbers of IFN- γ producing T cells in the lungs of CD40^{-/-} mice at 4 and 5 weeks, respectively (data not shown). Thus, the markedly improved survival of CD40^{-/-} mice is attributed to the induction of a stronger Th1 response characterized by a potent IFN- γ production in the lungs, solely as a result of higher antigen dose.

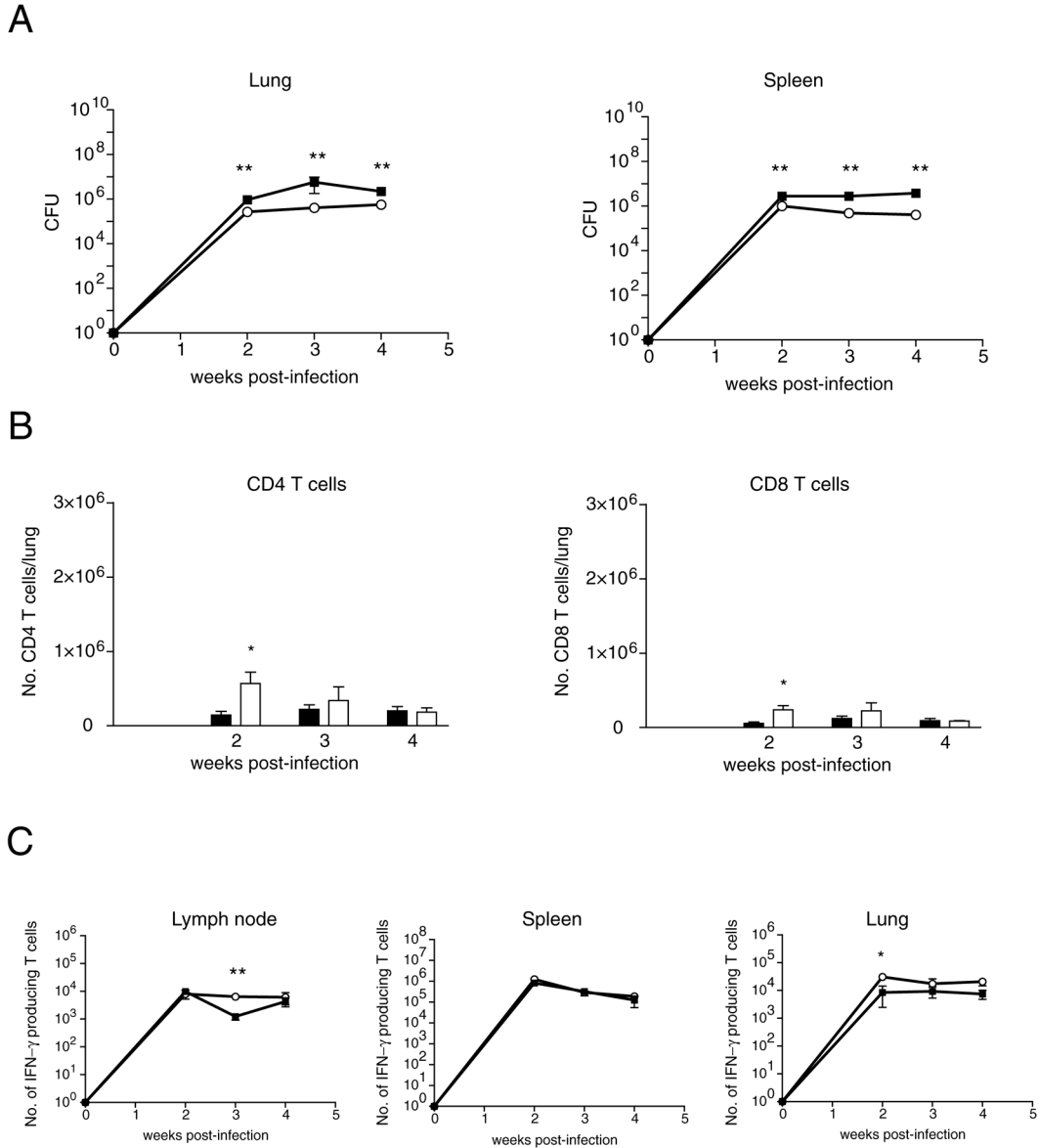


Figure 13 Infection of CD40^{-/-} mice with a higher dose of *M. tuberculosis* resulted in better priming of IFN- γ T cell responses

(A) CD40^{-/-} (■) and wild-type (○) mice were infected with 2×10^5 *M. tuberculosis* bacilli intravenously. At weekly time points, serial dilutions of lung and spleen homogenates were plated for CFU determination.

(B) The CD4⁺ and CD8⁺ T cell responses in the lungs of CD40^{-/-} (■) and wild-type (□) mice were analyzed using flow cytometry.

(C) 150,000 lymph node cells/well, 200,000 splenocytes/well and 80,000 lung cells/well from CD40^{-/-} (■) and wild-type (○) mice were incubated with uninfected or *M. tuberculosis*-infected wild-type dendritic cells for 40 hours in an ELISPOT assay. The number of IFN- γ producing T cells was calculated as follows: frequency \times total number of cells per lymph node, spleen or lung.

4.3.8. The outcome of *M. tuberculosis* infection in CD40^{-/-} and CD40L^{-/-} mice is different

The finding that IFN- γ responses were impaired in CD40^{-/-} mice after aerosol challenge was surprising as a previous study reported that CD40L^{-/-} mice developed mycobacteria-specific Th1 responses in the spleen and were resistant to intravenous infection with *M. tuberculosis* (212). This observation prompted us to investigate whether different routes of infection could influence the outcome of infection in CD40L^{-/-} mice. In contrast to CD40^{-/-} mice, CD40L^{-/-} or anti-CD40L-antibody treated mice were resistant to low dose (30 - 50 CFU) aerosol challenge with *M. tuberculosis* and controlled mycobacterial growth in the lungs and spleen comparably to control mice (Figure 14A and 14B, data not shown).

The magnitude of CD4⁺ and CD8⁺ T cell responses in the lungs of CD40L^{-/-} and anti-CD40L-antibody treated mice was similar to control mice, indicating that the development of protective T cell responses occurs normally in the absence of CD40L (Figure 14C, data not shown). As early as 2 weeks post-infection, there was a substantial number of IFN- γ secreting T cells in the lungs and lymph nodes of CD40L^{-/-} mice (Figure 14D). These results suggest that irrespective of the route of infection CD40L^{-/-} mice are resistant to *M. tuberculosis* challenge due to their ability to prime mycobacteria-specific Th1 T cells that migrate to the lungs early in infection, before the crisis point that we saw in CD40^{-/-} mice. The results obtained in mice treated with blocking anti-CD40L antibody correlated well with the results using CD40L^{-/-} mice, confirming that resistance in CD40L^{-/-} mice was not due to compensatory mechanisms in a knockout strain. These results suggest that CD40, but not CD40L, is required for the priming of IFN- γ T cell responses. Such asymmetry in the outcome of infection between CD40^{-/-} and CD40L^{-/-} mice points to the existence of an additional ligand for CD40. Wang *et al.* reported

that recombinant, LPS-free, *M. tuberculosis*-derived Hsp70 can ligate CD40 and induce chemokine, cytokine and NO expression by human dendritic cells, macrophages, and CD40 transfected cell lines (219, 220). We next tested the ability of recombinant, LPS-free, *M. tuberculosis*-derived Hsp70 to ligate CD40 on murine wild-type dendritic cells, and stimulate IL-12 production (Figure 15). Our results indicate that *M. tuberculosis*-derived Hsp70 induced significant production of IL-12 by wild-type dendritic cells, but not CD40^{-/-} dendritic cells, suggesting that *M. tuberculosis*-derived Hsp70 could be an alternative ligand for CD40.

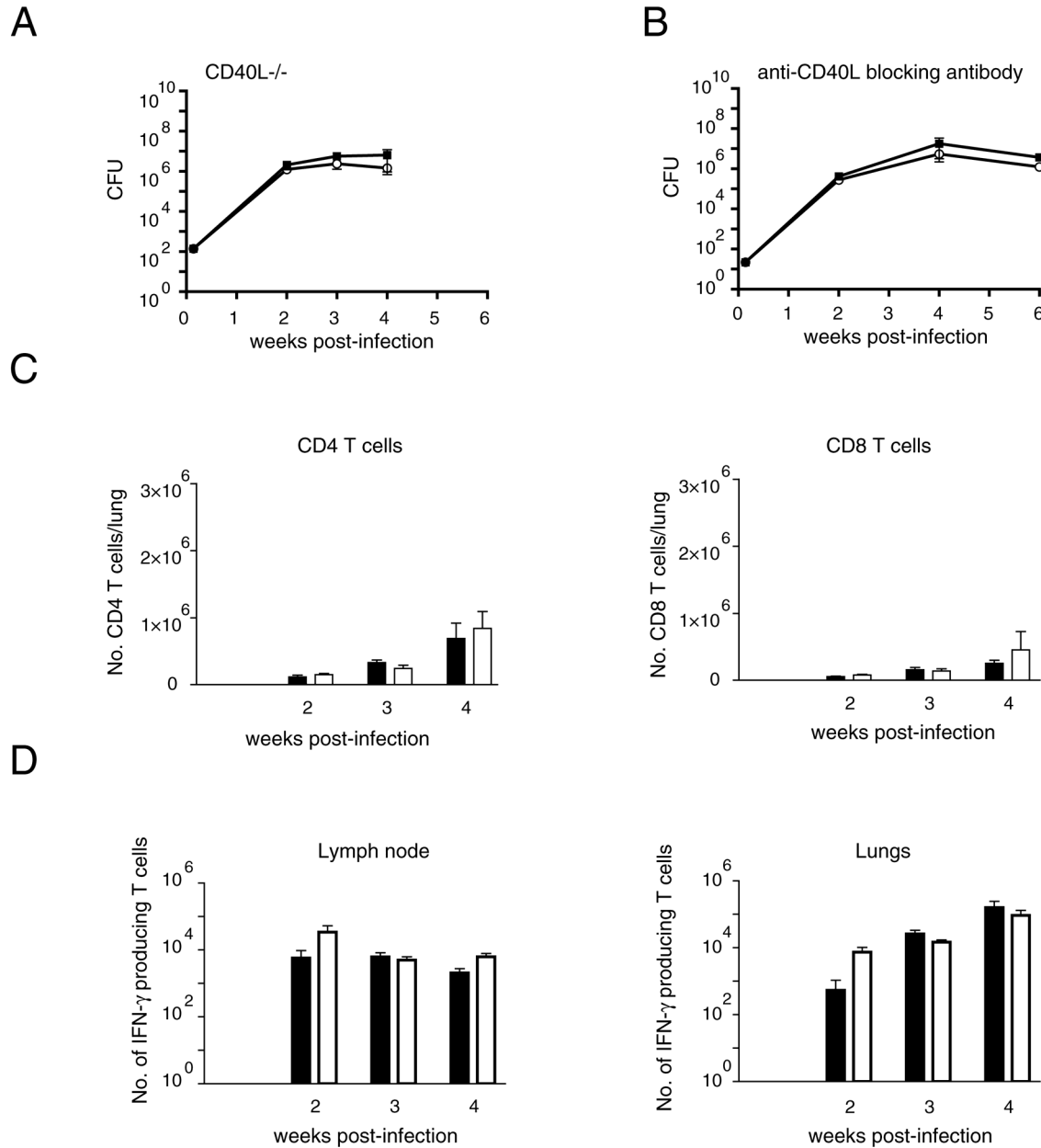


Figure 14 CD40L^{-/-} are able to control *M. tuberculosis* aerosol infection

(A and B) The number of colony forming units in the lungs of CD40L^{-/-}, anti-CD40L blocking antibody treated mice and their respective controls was determined by plating serial dilutions of lung homogenates on 7H10 plates.

(C) The number of CD4⁺ and CD8⁺ T cells in the lungs of CD40L^{-/-} and WT mice was determined by flow cytometry using the following formula = % total \times total number of cells per lung.

(D) The number of IFN- γ producing T cells was determined by ELISPOT as described in Materials and Methods. The results represent the mean of 3-4 mice per time point and error bars are standard error of mean. Statistical significance was determined by student t-test.

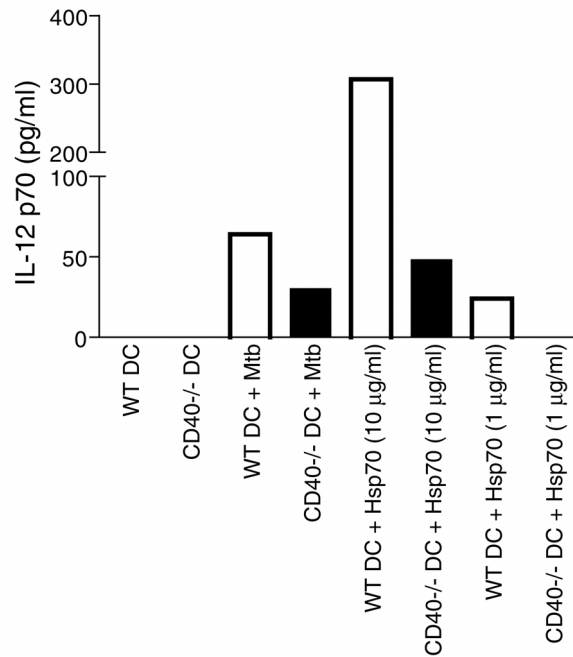


Figure 15 Asymmetry in the outcome of infection between CD40^{-/-} and CD40^{L-/-} mice could be due to existence of an alternative ligand for CD40 (*M. tuberculosis* derived Hsp70)

WT and CD40^{-/-} dendritic cells were left untreated, infected with *M. tuberculosis* (MOI 3), treated with 10 µg/ml or 1 µg/ml of recombinant, LPS-free, *M. tuberculosis*-derived Hsp70 for 24 hours. Supernatants were collected, filtered and the amount of IL-12 (p70) was estimated by ELISA.

4.4. Discussion

In this study we demonstrate that CD40 is essential for the induction of protective immunity against aerosol challenge with *M. tuberculosis*. The susceptibility of CD40^{-/-} mice to *M. tuberculosis* infection was attributed to the failure of these mice to rapidly mount protective Th1 responses. The underlying cause for poor induction of the Th1 response was due to deficient *in vivo* IL-12 production by CD40^{-/-} dendritic cells upon *M. tuberculosis* infection. Interestingly, the outcome of infection in CD40^{-/-} and CD40L^{-/-} mice was markedly different. This dichotomy implies that CD40 ligation, but not CD40L, is essential for the induction of a robust Th1 response in *M. tuberculosis* infection. In the absence of CD40L, stimulation through CD40 could be achieved by an alternative ligand. Here, we demonstrate that infection with *M. tuberculosis* or treatment with *M. tuberculosis* Hsp70 stimulates IL-12 production from wild-type dendritic cells in a CD40-dependent manner.

At 4 weeks post-infection, nearly 40% of CD40^{-/-} mice died, and histological analysis revealed excessive necrosis and consolidation of airspaces leading to hypoxia and respiratory distress in susceptible mice. However, some CD40^{-/-} mice lived for up to 5 months post-infection albeit with higher bacterial numbers in the lungs when compared with wild-type mice. This intriguing finding prompted us to determine the cause of susceptibility in CD40^{-/-} mice, and examine why a subset of CD40^{-/-} mice were capable of controlling *M. tuberculosis* infection.

Our results indicate that the susceptibility of CD40^{-/-} mice to *M. tuberculosis* infection stems from their inability to produce IFN- γ early in infection. This impaired Th1 response is related to inefficient priming of naïve T cells by CD40^{-/-} dendritic cells compared to wild-type dendritic cells. These results were surprising, as direct infection of APCs with a pathogen can be

sufficient to activate APC and overcome the need for co-stimulation in the induction of T cell responses (210). It is generally believed that infection of dendritic cells with *M. tuberculosis* is sufficient to mature them into potent antigen presenting cells. Infection of dendritic cells with *M. tuberculosis* results in up-regulation of antigen presenting, co-stimulatory and adhesion molecules, in production of inflammatory cytokines such as IL-1, IL-12 and TNF- α (112, 115, 116), and efficient *in vitro* priming of *M. tuberculosis*-specific CTL responses (138). Despite the fact that *M. tuberculosis*-infected CD40^{-/-} dendritic cells displayed phenotypic changes indicative of maturation, such as cell surface marker up-regulation, their ability to prime naïve T cells was diminished compared to wild-type dendritic cells. *M. tuberculosis*-infected wild-type dendritic cells produced greater than four times more IL-12 than infected CD40^{-/-} dendritic cells. Furthermore, attenuated *in vivo* IL-12 production may have a direct effect on the priming efficiency of Th1 responses in the lymph nodes of CD40^{-/-} mice. Exogenous administration of IL-12 to CD40^{-/-} DC – T cell co-cultures and to *M. tuberculosis*-infected CD40^{-/-} mice enhanced the priming of potent IFN- γ T cell responses.

Our results indicate that CD40^{-/-} mice manifested a major defect in the *in vivo* priming of *M. tuberculosis*-specific, IFN- γ T cell responses. This early defect in IFN- γ production in the lymph nodes of CD40^{-/-} mice had a significant impact on the magnitude of IFN- γ responses in the lungs. Infiltration of IFN- γ producing T cells into the lungs of CD40^{-/-} mice was substantially delayed compared with wild-type mice. While wild-type mice responded early to *M. tuberculosis* challenge with IFN- γ production, poor priming in CD40^{-/-} mice provided a window of opportunity for *M. tuberculosis* to overwhelm the immune system of CD40^{-/-} mice. These results underscore the importance of early IFN- γ production in resistance to *M. tuberculosis* infection as previously suggested with CD4⁺ T cell-deficient mice (126). *M.*

tuberculosis-infected CD40^{-/-} dendritic cells could re-stimulate T cells from infected wild-type mice to produce IFN- γ suggesting that the defect in CD40^{-/-} mice is at the level of priming, and that CD40 is not required for the effector function of T cells. *M. tuberculosis*-specific CD4⁺ T cell responses were more affected than CD8⁺ T cell responses by the absence of CD40 molecule. This finding was surprising as it is believed that CD4⁺ T cell help for the development of CD8⁺ T cell responses is mediated through CD40/CD40L interaction (210, 221, 222).

Direct comparison of gene expression between CD40^{-/-} mice that were moribund and those that were controlling the infection at 4 weeks revealed a striking difference in the amount of IFN- γ mRNA present in the lungs. While moribund CD40^{-/-} mice had undetectable IFN- γ mRNA levels, CD40^{-/-} mice that controlled infection showed wild-type levels of IFN- γ gene expression. These results support the conclusion that the ability of surviving CD40^{-/-} mice to control *M. tuberculosis* challenge is due to successful induction of IFN- γ production.

It has been proposed that the strength of signal and duration of TCR stimulation can determine the level of co-stimulation required to achieve efficient T cell activation (217, 218). High antigen dose and prolonged TCR ligation were shown to overcome the need for co-stimulation in the induction of T cell responses (223). CD40^{-/-} mice did not exhibit a permanent impairment, but rather a 4 week-delay, in IFN- γ production in the lungs. This suggests that continuous replication of mycobacteria within the lungs of CD40^{-/-} mice may generate a sufficient antigen dose that will elicit repeated and prolonged TCR stimulation, thereby overcoming the need for CD40/CD40L interaction as proposed for some viral infections (reviewed in (218)). Thus, those mice that survived the critical point may have received a slightly higher dose of bacteria during aerosol infection, or supported a higher antigen load early in the lungs.

To evaluate the effect of antigen dose and systemic infection on CD40 dependent induction of Th1 responses, we infected CD40^{-/-} mice with a higher bacterial inoculum intravenously and via aerosol. High dose intravenous and aerosol infections resulted in improved induction of IFN- γ responses, maintenance of stable bacterial numbers in the lungs and spleens, and survival of mice throughout duration of the study. These results suggest that high antigen dose and priming at multiple sites achieved through systemic infection or immunization may overcome the need for CD40 ligation in the induction of mycobacteria-specific IFN- γ responses.

Activation of macrophages was not obviously impaired by the absence of CD40/CD40L interaction. Production of RNI appears to be a primary effector mechanism by which activated macrophages contribute to the growth inhibition and killing of intracellular mycobacteria (215). Induction of NOS2, as an indicator of macrophage activation, can be achieved by soluble mediators, such as IFN- γ and TNF- α , or through T cell-macrophage contacts via CD40-CD40L interaction (215, 224). CD40^{-/-} macrophages had no intrinsic defect in their ability to reduce the numbers of intracellular bacteria or produce RNI after *in vitro* LPS/IFN- γ or T cell-mediated activation. Moreover, lung sections from CD40^{-/-} mice did not reveal any obvious differences in NOS2 expression, suggesting that CD40/CD40L interaction is not necessary for induction of NOS2 *in vivo*. Although CD40^{-/-} mice have significantly reduced amounts of IFN- γ and lack the CD40 molecule, these mice have normal NOS2 mRNA and protein expression. These results suggest that either there is another pathway for induction of NOS2, independent of IFN- γ and/or CD40/CD40L interaction, or the reduced levels of IFN- γ in the lungs of CD40^{-/-} mice were sufficient to activate macrophages through a conventional IFN- γ dependent pathway. It remains unclear as to why CD40^{-/-} mice still succumb to *M. tuberculosis* infection despite normal levels of NOS2. Although induction of NOS2 and production of RNI intermediates are absolutely

essential for protection against *M. tuberculosis* (105, 215), they may not be sufficient to protect CD40^{-/-} mice in the absence of optimal IFN- γ production. We previously reported that chronically infected mice succumbed to infection after depletion of CD4⁺ T cells, even though IFN- γ and NOS2 production in the lungs was unaffected (48). These data emphasize the importance of NOS2- and IFN- γ - independent anti-mycobacterial mechanisms in control of tuberculosis.

The susceptibility of CD40^{-/-} mice to low dose aerosol challenge was unexpected, as a previous study reported that CD40L^{-/-} mice were resistant to intravenous *M. tuberculosis* infection (212). To exclude the possibility that the route of infection could have an effect on the outcome of infection, we infected CD40L^{-/-} and wild-type mice that were treated with the blocking anti-CD40L antibody with a low dose of *M. tuberculosis* by aerosol. CD40L^{-/-} mice were resistant to *M. tuberculosis* infection irrespective of the infection route. The development of Th1 responses in CD40L^{-/-} and anti-CD40L antibody treated mice was similar to control mice, indicating that the absence of CD40L has negligible impact on the generation of IFN- γ responses in *M. tuberculosis* infection. We noted a minor difference in the number of IFN- γ producing cells in the lungs and lymph nodes of CD40L^{-/-} mice at 2 weeks post-infection. However, this difference did not influence the outcome of infection as it occurred at the time when the CFU in CD40L^{-/-} mice were still low. By 3 weeks post-infection CD40L^{-/-} mice exhibited strong lung IFN- γ responses comparable to wild-type mice resulting in stable control of mycobacterial burdens.

These data indicate that CD40, but not CD40L, is required for the optimal priming of T cells and control of *M. tuberculosis* infection. One possible explanation could be the existence of another host-derived ligand for CD40, as shown for other members of TNF-R superfamily (198,

225). Alternatively, a *M. tuberculosis*-encoded protein may directly ligate CD40 on APCs, thereby modulating their function. Our results support that a *M. tuberculosis*-derived product induces IL-12 production by dendritic cells through CD40 ligation. In the absence of exogenous IFN- γ or T cells, the level of IL-12 produced by *M. tuberculosis*-infected wild-type dendritic cells was four times greater than the amount of IL-12 produced by *M. tuberculosis* infected CD40^{-/-} dendritic cells. Furthermore, we found that recombinant *M. tuberculosis* Hsp70 stimulated significant IL-12 production by wild-type but not CD40^{-/-} dendritic cells, suggesting that Hsp70 is an alternative ligand for CD40. A recent publication reported that mycobacterial heat shock protein 70 (Hsp70) binds to CD40 and stimulates human mononuclear cells to release CC-chemokines RANTES, MIP-1 α , and MIP-1 β (219). Thus, our data and this publication strongly support a role for mycobacterial derived proteins in the stimulation of CD40 for priming immune responses.

One question is why would a pathogen evolve to induce strong T cell responses? Granulomas are formed following T cell induction in *M. tuberculosis* infection; these granulomas contain, but do not always eliminate the organisms. One could speculate that granuloma formation, which is dependent on T cell responses, is essential for effective transmission of *M. tuberculosis* infection. Transmission is greatly enhanced in persons with cavitation, i.e. a granuloma reactivating and breaking through a bronchus, releasing large numbers of bacteria into the airways. This might explain why *M. tuberculosis* would maintain molecules that strongly stimulate induction of a T cell response.

Our initial interest in CD40/CD40L interaction stemmed from experiments in which CD4⁺ T cell depletion in chronically infected mice resulted in the death of mice despite normal levels of IFN- γ and NOS2 expression in macrophages (48). In addition, CD4^{-/-} and MHC Class

II-/- mice succumb to acute *M. tuberculosis* infection despite the compensatory IFN- γ production by CD8⁺ T cells in the lungs (126, 138). Since CD40L is primarily expressed by activated CD4⁺ T cells, we sought to determine whether additional roles of CD4⁺ T cells in the control of *M. tuberculosis* infection are mediated through CD40/CD40L interaction. Our results show that the phenotype of CD40-/- mice differs considerably from that of CD40L-/- and CD4-/- mice (data not shown) indicating that CD40/CD40L interaction is not the mechanism by which CD4⁺ T cells contribute to the control of acute or chronic *M. tuberculosis* infection.

In conclusion, our results demonstrate the importance of CD40 in the generation of protective immunity against *M. tuberculosis*. The failure of CD40-/- mice to control *M. tuberculosis* infection is attributed to inefficient priming of IFN- γ T cell responses. CD40 dependence on induction of IFN- γ responses appears to be a function of antigen dose as intravenous and aerosol infection of CD40-/- mice with a higher dose of *M. tuberculosis* overcomes the need for co-stimulation in the induction of IFN- γ responses. These results may have important implications for vaccine development and priming of immune responses in humans following *M. tuberculosis* infection as the rate at which T cell responses are primed could influence progression to disease or containment of the infection.

5. CHAPTER 2 LONG-TERM CONTROL OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION IS MEDIATED BY DYNAMIC IMMUNE RESPONSES

5.1. Introduction

Mycobacterium tuberculosis remains the largest cause of death in the world attributed to a single infectious agent (226). Over the past twenty years, work utilizing gene-knockout murine models has proven essential for understanding the contributions of various immune cell types and effector arms in the successful long-term control of *M. tuberculosis* infection (reviewed in (227)). IL-12 production by *M. tuberculosis*-infected dendritic cells is essential for the priming of potent Th1 responses characterized by IFN- γ and TNF production by CD4⁺ and CD8⁺ T cells (119, 228). The activation of these responses culminates in the formation of organized structures in the lungs called granulomas. Elaboration of proinflammatory cytokines by T cells leads to induction of bacteriostatic and bactericidal mechanisms by infected macrophages mediated via RNI and ROS pathways (reviewed in (227)). Coincident with the onset of *M. tuberculosis*-specific T cell mediated immunity, bacterial growth is suppressed and maintained under strict control, resulting in life-long containment and latency within infected individuals. However, in 10% of infected people spontaneous reactivation occurs, usually as a consequence of waning immune function. This risk of reactivation is increased in HIV⁺ infected individuals to 10% per year, emphasizing the role of CD4⁺ T cells in restraining *M. tuberculosis* growth and maintaining latency (128).

It has been particularly challenging to establish adequate animal models of human latent tuberculosis. Mice infected with *M. tuberculosis* typically do not display overt signs of disease (45), and reactivation occurs when experimental manipulations such as CD4 depletion, nitric

oxide synthase inhibition or TNF neutralization are implemented (46-49). Unlike humans, bacterial loads are maintained at relatively high levels ($\sim 1 \times 10^6$ CFU per lung), which can lead to progressive immunopathology. Therefore, the murine model of tuberculosis is more reflective of persistent *M. tuberculosis* infection, a feature which can be exploited to study the influence of chronic exposure to antigen on effector functions of *M. tuberculosis*-specific $CD4^+$ and $CD8^+$ T cells.

It has been shown in viral persistent infections caused by human immunodeficiency virus (HIV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), Friend virus (FV) and Clone 13 lymphocytic choriomeningitis virus (LCMV) infections that chronic exposure of $CD8^+$ T cells to viral antigens leads to functional abnormalities including impaired cytotoxicity and cytokine production, reduced proliferative capacity, and clonal deletion or exhaustion (229-237). This dysregulation of effector capabilities is not limited to $CD8^+$ T cells. Infection with high dose of LCMV Clone 13 leads to aberrant $CD4^+$ T cell responses marked by reduced IFN- γ and IL-2 production (233, 234). From these findings a model has emerged in which chronic exposure to high viral load culminates in functionally weak $CD4^+$ T cell responses. Inadequate $CD4^+$ T cell help concomitant with chronic exposure to high antigen doses can generate defective $CD8^+$ T cell responses leading to persistent viral infection (233, 234).

Therefore, the role of antigen levels on shaping the functional competency and developmental skewing of T cell responses has become recognized as a key factor in chronic disease processes. For example, the removal of antigen stimulation can lead to reacquisition of effector functions, and high or low antigen states have been shown to be primary determinant of the cytokine versus cytolytic nature of virus specific $CD8^+$ T cell responses (233, 238, 239).

In this study we sought to determine the effect of prolonged exposure to high bacterial loads on the quality of effector CD4⁺ and CD8⁺ T cell responses during a 5-7 month period of *M. tuberculosis* infection. The results from our study reveal several important findings: (i) CD4⁺ and CD8⁺ T cells did not show signs of replicative senescence, but rather demonstrated two bursts of expansion during a 7 month course of infection; (ii) although CD4⁺ T cells were the primary source of IFN- γ during acute infection, CD8⁺ T cells equally contributed to IFN- γ production during chronic infection; (iii) the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells dynamically changed during protracted *M. tuberculosis* infection; and (iv) there was differential regulation of CD8⁺ T cell effector functions during different phases of infection whereupon CD8⁺ T cells functioned primarily as cytotoxic T lymphocytes during acute infection, and switched to cytokine production during chronic infection. This lack of CTL activity was not accompanied by loss of perforin production. Collectively, our findings depict a dynamic host immune response during persistent *M. tuberculosis* infection characterized by quantitative and qualitative differences in the effector functions of CD4⁺ and CD8⁺ T cell responses that were previously unrecognized.

5.2. Materials and Methods

5.2.1. Mice and infections

C57BL/6 wild-type mice were purchased from Charles River Laboratories (Wilmington, MA). All mice were kept under specific pathogen-free conditions in a Biosafety Level 3 facility, and animal protocols were approved by the University Institutional Animal Care and Use Committee. Mice were infected with a low dose of *M. tuberculosis* (Erdman strain, Trudeau Institute, Saranac Lake, NY) at 5×10^5 /ml using a nose-exposure only aerosolizer unit (Intox Inc., Moriarty, NM). The dose received was estimated by plating whole lung homogenates of three mice 24 hours following aerosol infection. Twenty mice were used for each time point, with 5 mice per experimental group.

5.2.2. CFU determination

Lung homogenates were serially diluted in PBS/0.05% Tween-80 and plated on 7H10 agar plates (Difco). Plates were incubated at 37°C, 5% CO₂ for 21 days prior to counting colonies.

5.2.3. Bone marrow derived macrophages and dendritic cell cultures

In *ex vivo* stimulation assays, such as ELISPOT and limiting dilution analysis, bone marrow derived dendritic cells were cultured in the presence of GM-CSF supernatant at 1:200 dilution (a generous gift from Dr. Binfeng Lu, University of Pittsburgh) and 20 ng/ml of IL-4 (PeproTech Inc, Rocky Hill, NJ) and macrophages were cultured in the presence of L cell sup as a source of CSF-1 using standard procedure described previously (146).

5.2.4. Preparation of lung cells

For all experiments performed in this study, we treated lung cells with 1 mg/ml of collagenase A and 25 U/ml of DNase (Roche, Mannheim, Germany) at 37°C for 20 minutes. The lungs were crushed with a 5 ml syringe plunger through 70 µm cell strainer to obtain single cell suspension. Red blood cells were lysed with NH₄Cl/Tris solution for 2 minutes at room temperature. Following one wash with 1x PBS, cells were suspended in T cell media (DMEM, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 25 mM HEPES, 50 µM 2-ME [Sigma]) and counted using trypan blue exclusion method.

5.2.5. Flow cytometry

Lung single cell suspensions were stained as described previously (180). Cells were stained with anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7), anti-CD69 (clone H1.2F3), anti-Ly6C (clone AL-21), anti-CD44 (clone IM7), and anti-CD62L (clone MEL-14) fluorescently conjugated antibodies. All antibodies were purchased from BD Pharmingen (San Diego, CA) and used at 0.2 µg/ml concentration. Cells were collected on a FACSCaliber (Beckon Dickinson, San Jose, CA) and analyzed by CellQuest (Becton Dickinson) or FlowJo (Tree Star Inc, San Carlos, CA) software.

5.2.6. Proliferation of T cells in the lungs of infected mice

Sixteen hours prior to each experimental time point, mice were injected intraperitoneally with saline containing 1 mg of 5-bromo-2'-deoxyuridine [BrdU] (Sigma-Aldrich, St. Louis, MO). Lung cells were stained for cell surface markers CD4 and CD8 at room temperature for 20 minutes prior to a fixation step with 4% paraformaldehyde [PFA] (200 µl/tube) on ice for 20

minutes. Cells were washed with tissue culture phosphate buffered saline [PBS] at 470 g, and cell pellets were suspended in ice-cold 0.15 M NaCl (100 µl/tube), followed immediately by drop-wise fixation with ice-cold 95% ethanol (200 µl/tube) on ice for 30 minutes. Following a PBS wash, cells were permeabilized and fixed with 200 µl/tube of 0.4% saponin and 2% PFA for 1 hour at room temperature. Cells were washed with PBS, and suspended in 200 µl/tube of 0.15 M NaCl, 4.2 mM MgCl₂ (pH 5) containing 250 U/ml of DNase I (Roche, Indianapolis, IN) for 30 minutes in 37°C water bath. Cells were washed with PBS, and incubated with anti-BrdU antibody or the respective isotype control (BD Pharmingen, FITC-conjugated antibody set) diluted at 1:3 in 0.5% Tween-20 and 0.5% BSA (50 µl/tube). Following 30 minute incubation at room temperature, cells were washed with PBS, and fixed with 4% PFA prior to acquisition on the flow cytometer.

5.2.7. Apoptosis staining

Lung cells were stained for the expression of CD4 and CD8 molecules for 20 minutes at room temperature. The amount of apoptosis was determined by staining lung cells with Annexin V-FITC and 7-AAD reagents (BD Pharmingen) according to manufacturer's instructions. Briefly, cells were suspended in 100 µl/tube of 1x Binding buffer, and incubated with 5 µl/tube of Annexin V-FITC and 5 µl/tube of 7-AAD for 15 minutes in the dark at room temperature. Cells were washed with 2 ml/tube of 1x Binding buffer to remove any unbound Annexin V-FITC and 7-AAD, and fixed with 4% PFA in 1x Binding buffer. Samples were analyzed within 30 minutes.

5.2.8. IFN- γ production

Cytokine production by CD4⁺ and CD8⁺ T cells isolated from the lungs of *M. tuberculosis* infected mice was evaluated by intracellular cytokine staining and ELISPOT as described previously (130, 228). Briefly, for intracellular cytokine staining, lung and lymph node cells were incubated in media or stimulated with anti-CD3 (BD Pharmingen, clone 145-2C11, 0.1 μ g/ml) and anti-CD28 (BD Pharmingen, clone 37.51, 1 μ g/ml) antibodies for 4 hours in the presence of 3 μ M monensin (Sigma-Aldrich, St. Louis, MO). At the end of the stimulation period, cells were stained for CD4 and CD8, fixed, permeabilized, and stained for IFN- γ expression.

For ELISPOT, lung and lymph node cells were plated in anti-IFN- γ antibody (BD Pharmingen, clone R4-6A2) coated plates (MAIPS4510, Millipore Corp, Bedford, MA) at 80,000 cells/well and 150,000 cell/well, respectively. Cells were incubated in duplicate wells with media, ConA (10 μ g/ml; Sigma-Aldrich), uninfected and *M. tuberculosis*-infected dendritic cells (MOI 3; overnight) to estimate the number of total number of IFN- γ producing T cell, and *M. tuberculosis*-infected dendritic cells incubated with the blocking anti-MHC Class I (BD Pharmingen, clone 8F12) or anti-MHC Class II (BD Pharmingen, clone M5/114.15.2) antibodies at 10 μ g/ml to estimate the number of IFN- γ producing CD4⁺ and CD8⁺ T cells, respectively. In addition, dendritic cells were pulsed with ESAT-6 protein (received from NIH Tuberculosis Research Reagent Contract NO1 AI-75320) at 10 μ g/ml overnight. All dendritic cells were added to lung and lymph nodes cells at 1:2 ratio and the cultures were supplemented with IL-2 (PeproTech) at final concentration of 20 U/ml. Following 40 hour incubation, the IFN- γ producing T cells were visualized after stepwise incubation of plates with biotinylated anti-IFN- γ antibody (BD Pharmingen, clone XMG 1.2) , streptavidin-conjugated enzyme (PK-6100, Vector

Laboratories) and AEC substrate (SK-4200, Vector Laboratories). The spot forming units (SFU) were enumerated using ELISpot reader (Cellular Technology Ltd, Cleveland, OH).

5.2.9. Limiting dilution analysis

The cytotoxic potential of CD8⁺ T cells was estimated using limiting dilution analysis (LDA) (240, 241). Effector cells were derived from the lungs and lung draining lymph nodes of *M. tuberculosis*-infected mice at designated time points. Freshly isolated cells were plated in 2-fold serial dilutions starting from 40,000 cells/well to 1250 cells/well in V-bottom 96-well plates (24 replicates/input number) supplemented with IL-2 at 20 U/ml. Lung and lymph node cells were incubated with *M. tuberculosis*-infected dendritic cells (500 DC/well) for 7 days. Following incubation, 100 µl of old media was removed from each well, and lung cells were cultured for another round of stimulation with *M. tuberculosis*-infected macrophages (1000 MΦ/well) and IL-2 (20 U/ml) to allow for expansion of CTL precursors. Flow cytometry analysis revealed that 75-95% of cells were CD8⁺ T cells after 2 weeks of stimulation.

Cytotoxicity was determined in each well by a standard ⁵¹Chromium release assay with *M. tuberculosis*-infected macrophages as targets. *M. tuberculosis*-infected macrophages were labeled with ⁵¹Cr (100 µl of ⁵¹Cr per 3x10⁶ macrophages) for 1 hour at 37°C, and added to lung and lymph node T cell cultures at 4000 cells/well. Following 4 hour incubation, 100 µl of supernatant was collected (Skatron SCS System; Skatron, Sterling, VA) and radioactivity was quantified using a gamma counter. Positive wells were defined as being greater than mean + 3 SD of spontaneous target cell release. Frequency of CTLp was determined using zero-order Poisson equation ($\ln Y = -Fx + \ln A$; where x = the number of effector cells/well; Y = % negative wells; A = the y-axis intercept; F = CTLp frequency defined by the negative slope of

the line). All calculations were performed using a software program fitted to the equation by χ^2 minimization analysis (a generous gift from Dr. Carolyn A. Keever-Taylor, Medical College of Wisconsin).

5.2.10. Intracellular perforin and IFN- γ staining

To determine perforin and IFN- γ expression within CD8⁺ T cells we adapted the technique for combined intracellular staining of perforin and IFN- γ as previously described by Slifka *et al.* (242). Freshly isolated lung and lymph node cells were incubated with the stimulating anti-CD3 (BD Pharmingen, clone 145-2C11, 0.1 μ g/ml) and anti-CD28 (BD Pharmingen, clone 37.51, 1 μ g/ml) antibodies for 4 hours in the presence of 3 μ M monensin (Sigma-Aldrich, St. Louis, MO). At the end of the stimulation period, cells were fixed with 2% PFA for 20 minutes on ice, washed, and permeabilized with 0.1% saponin/1%FBS/ Ca²⁺-free PBS. Cells were incubated with 1:200 dilution of anti-perforin antibody (clone KM 585 (P1-8), Kamiya Biomedical) in 0.3% saponin/5% normal goat serum/Ca²⁺-free PBS (50 μ l/tube) at 4°C for 30 minutes. Cells were washed with 0.1% saponin, and incubated with the secondary goat anti-rat IgG-FITC antibody (BD Pharmingen) at 4°C for 30 minutes. Following incubation, cells were stained with anti-IFN- γ PE (BD Pharmingen, clone XMG 1.2) and anti-CD8⁺ CY (BD Pharmingen, clone 53-6.7) antibodies for 20 minutes at room temperature. Cells were washed with 0.1% saponin, and suspended in 2% PFA until flow cytometry analysis.

5.2.11. Statistics

Statistically significant differences in the numbers of effector T cells between the two time points were determined using unpaired, two-sided, Student t-test. The p-value of < 0.05 was defined as being significant.

5.3. Results

5.3.1. Generation of immune response follows bacterial replication kinetics

Following a low dose aerosol infection ($\sim 20 - 50$ CFU/lung), *M. tuberculosis* replicated exponentially in the lungs of infected mice, peaking 3 weeks post-infection and then stabilizing at 4 weeks post-infection (Figure 16A). Enhanced replication of mycobacteria early in infection was followed by increased infiltration of immune cells into the lung, which peaked and stabilized at 4 weeks post-infection (Figure 16B). This control of *M. tuberculosis* replication coincided with the formation of organized structures called granulomas consisting of T cells, B cells, macrophages and dendritic cells (data not shown). Thus, with the induction of immune response, growth of mycobacteria was hindered and maintained under control during long-term persistent infection.

To determine the kinetics of CD4⁺ and CD8⁺ T cell responses during acute and chronic infection, lungs were crushed into single cell suspensions and stained for the expression of CD4 and CD8 molecules. Unlike many well-studied viral or bacterial infections, there does not seem to be an immunodominant antigen recognized by murine CD8⁺ T cells in *M. tuberculosis* infection. Although there is a dominant *M. tuberculosis* antigen recognized by CD4⁺ T cells early in infection ((132) and see below), MHC Class II multimer reagents for this antigen are not readily available. These limitations preclude the identification of *M. tuberculosis* antigen-specific T cells by flow cytometry. In this section we addressed the complete T cell population in the lungs during *M. tuberculosis* infection. Since the lung is not a lymphoid organ, the majority of cells observed are present due to the infection.

The number of CD4⁺ and CD8⁺ T cells in the lungs increased 10-fold from 2 to 4 weeks post-infection. The number of CD4⁺ T cells was 2-fold higher than the number of CD8⁺ T cells during the peak of the response, which was followed by a retraction phase in both T cell populations once bacterial numbers stabilized (Figure 16C and 16D). During chronic infection the numbers of both bacteria and immune cells were maintained at a steady state level.

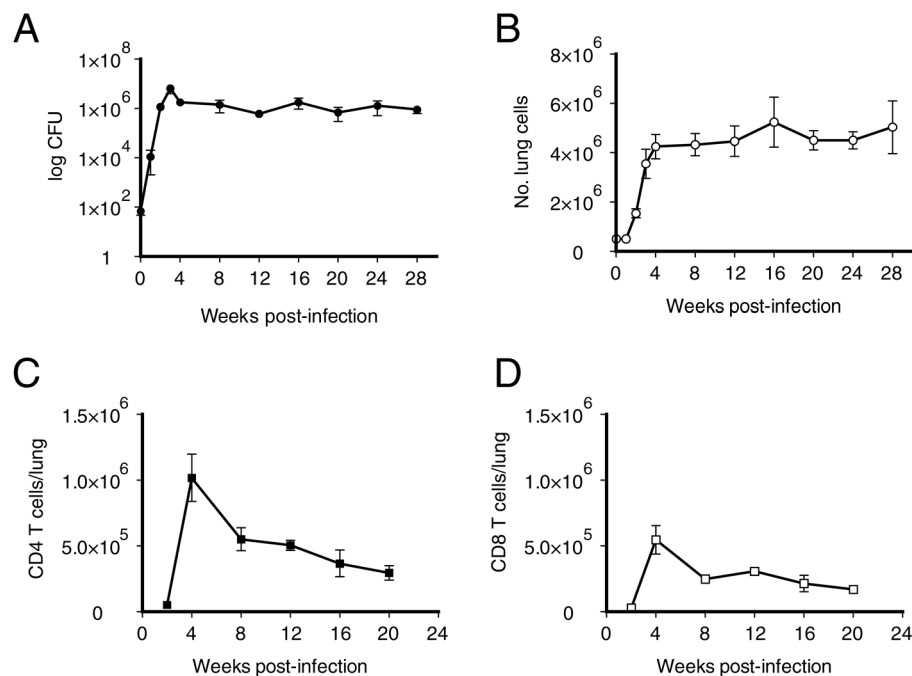


Figure 16 Infiltration of immune cells into the infected lungs follows the bacterial replication kinetics. CD4⁺ and CD8⁺ T cells follow programmed retraction phase after acute *M. tuberculosis* infection

(A) The number of live mycobacteria was determined by plating serial dilution of lung homogenates on 7H10 plates. Following 21 days of incubation, colonies were enumerated, and the number of CFU per lung determined.

(B) The number of live cells in the infected lungs was quantified using trypan blue exclusion method.

(C and D) The number of CD4⁺ and CD8⁺ T cells was quantitated after staining lung cells with anti-CD4 and anti-CD8 antibodies using flow cytometry. The number of cells was calculated by multiplying the percent of CD4⁺ and CD8⁺ positive cells within live cell gate and the total number of live cells in the lungs. The data represent the mean \pm SEM of 8 – 20 mice per time point.

5.3.2. Turnover of CD4⁺ and CD8⁺ T cells in the lungs of *M. tuberculosis* infected mice

To gain insights into the turnover of CD4⁺ and CD8⁺ T cells during acute and chronic infection, we measured the amount of proliferation and apoptosis using BrdU incorporation and annexin V – 7-AAD staining, respectively. BrdU was injected 16 hours prior to each time point to obtain an accurate estimation of *in situ* proliferating cells. Lung cells were harvested at indicated time points, and cells from the same mice were stained either with anti-CD4, anti-CD8 and anti-BrdU antibodies; or with annexin V, 7-AAD, anti-CD4 and anti-CD8 antibodies to quantify proliferating and apoptotic cells, respectively.

Figure 17A depicts the percentage of BrdU⁺ CD4⁺ and BrdU⁺ CD8⁺ T cells within the lymphocyte gate. Initially during the peak of the response there was a 6-fold increase in the percentage of BrdU⁺ CD4⁺ and BrdU⁺ CD8⁺ T cells. Once bacterial numbers stabilized, the percentage of proliferating cells decreased and remained low until late stage of infection. Apoptosis occurring within CD4⁺ and CD8⁺ T cell populations followed similar kinetics as BrdU incorporation: there was an initial increase in the percentage of apoptotic cells during the peak of the response followed by a stabilization phase with more apoptotic CD4⁺ than CD8⁺ T cells. The amount of apoptosis increased during the late stage of chronic infection (Figure 17B).

The graphs in Figure 17C and 17D directly compare the amount of proliferation and apoptosis within CD4⁺ and CD8⁺ T cell gates. These experiments provide three important results. First, the 10-fold increase in the numbers of CD4⁺ and CD8⁺ T cells during the peak of response at 4 weeks post-infection (Figure 16C and 16D) was mainly due to infiltration of primed effector T cells into the inflamed lung rather than *in situ* proliferation of the effector cells, as only 5% of CD4⁺ and CD8⁺ T cells were proliferating at this time point (Figure 17C and 17D). Second, the

retraction phase was brought about by increased apoptosis and reduced proliferation of T cells as infection progressed into the chronic phase. Third, the initial wave of proliferation was followed by another burst of proliferation of a smaller magnitude within CD4⁺ and CD8⁺ T cell populations at 5 months post-infection (Figure 17C and 17D).

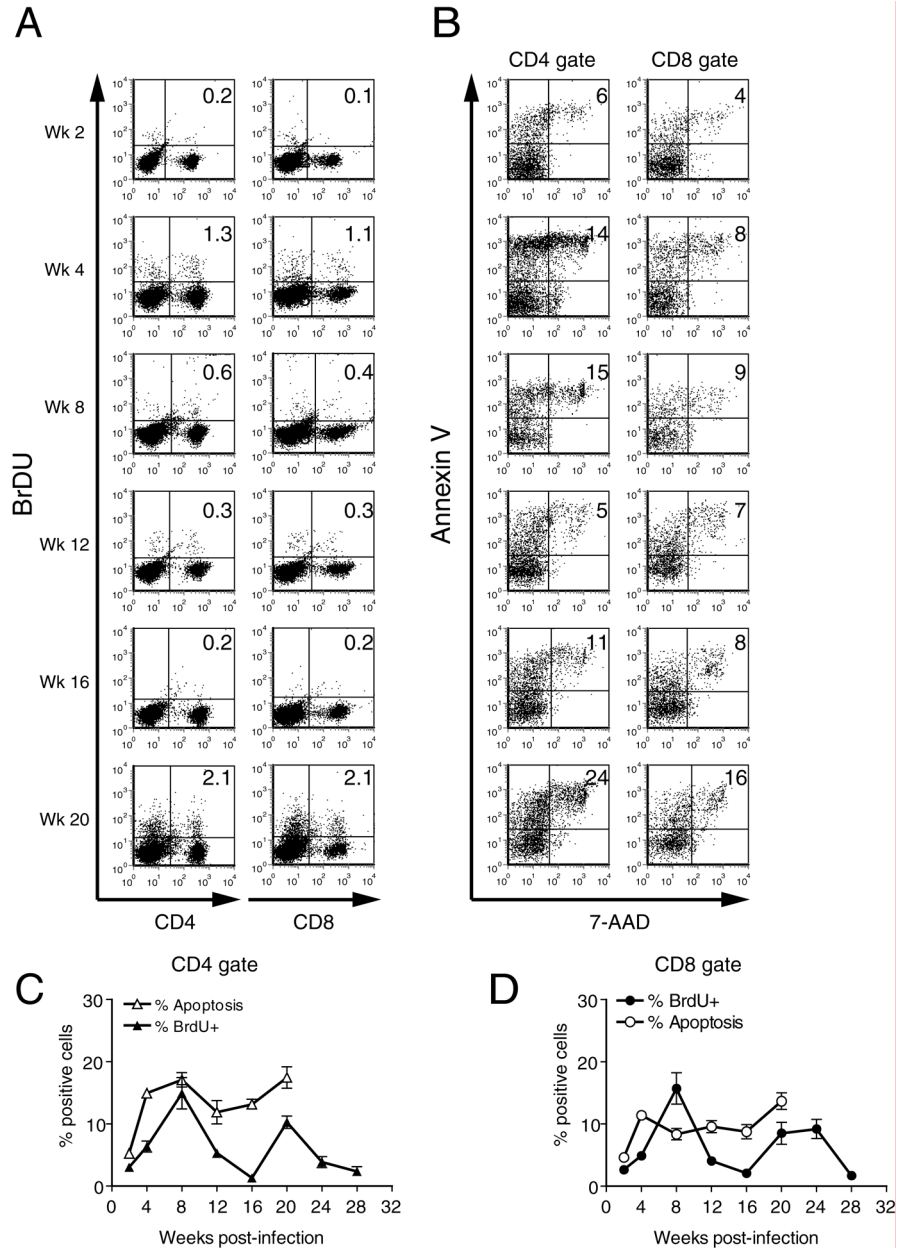


Figure 17 Turnover of CD4⁺ and CD8⁺ T cells in the lungs of *M. tuberculosis* infected mice

(A) The percentage of CD4⁺BrdU⁺ and CD8⁺BrdU⁺ cells within lymphocyte gate was determined following a 16 hour injection of BrdU (1 mg/mouse) using flow cytometry.

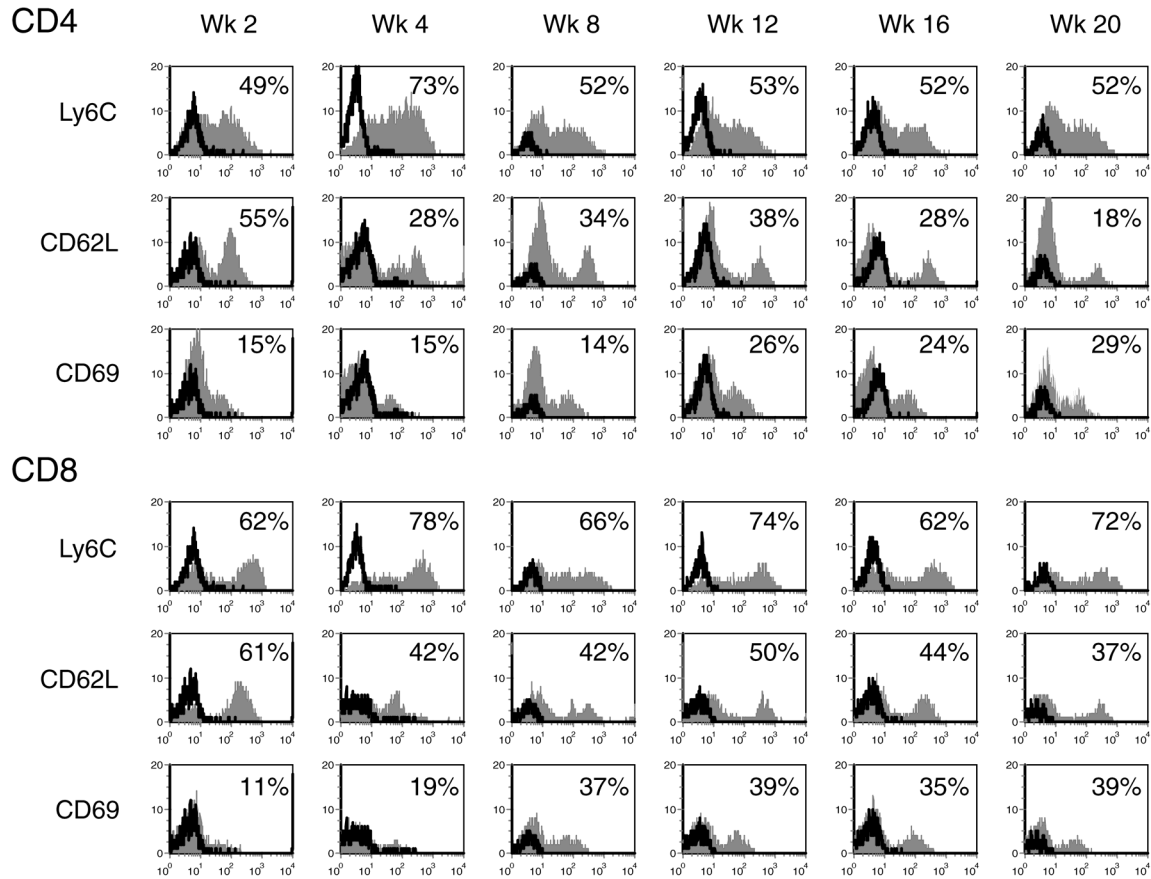
(B) The amount of apoptosis within the lungs of infected mice was assessed following staining of lung cells with annexin V and 7-AAD. Only double positive cells were considered to be apoptotic.

(C and D) The graphs summarize the percentage of proliferating and apoptotic cells within CD4⁺ and CD8⁺ gates. The data represent mean \pm SEM of 5 mice per time point.

5.3.3. CD4⁺ and CD8⁺ T cells display activated effector phenotype in the face of chronic *M. tuberculosis* infection

To determine the activation status of CD4⁺ and CD8⁺ T cells during acute and chronic infection, we isolated cells from the lungs of infected mice at indicated time points and stained cells for the expression of CD4, CD8, Ly6C, CD62L and CD69 (Figure 18A). The vast majority of CD4⁺ and CD8⁺ T cells isolated from the lungs of infected mice displayed high levels of CD44 as early as 2 weeks post-infection (data not shown), up-regulated and remained Ly6C high, down-regulated CD62L and up-regulated CD69 (Figure 18B). Although CD8⁺ T cells were present in fewer numbers than CD4⁺ T cells, the percentage of CD69⁺ CD8⁺ T cells was more than 1.5-fold higher when compared to CD69⁺ CD4⁺ T cells during chronic infection, indicating that more CD8⁺ T cells were in immediate contact with infected cells (and thus stimulated by MHC molecules) inside the lungs (Figure 18A and 18B). These data demonstrate the presence of highly activated CD4⁺ and CD8⁺ T cells during persistent *M. tuberculosis* infection.

A



B

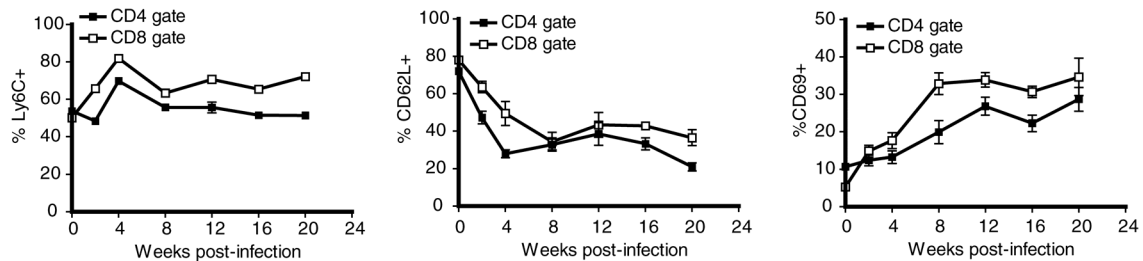


Figure 18 CD4⁺ and CD8⁺ T cells exhibit activated phenotype in the lungs during persistent *M. tuberculosis* infection

(A) Single lung cell suspensions were stained with anti-CD4, anti-CD8, anti-Ly6C, anti-CD62L and anti-CD69 antibodies and the relative expression of the indicated markers within CD4⁺ and CD8⁺ gates (filled histograms) was estimated by flow cytometry. The solid line represents background staining with isotype control antibody.

(B) The graphs illustrate the percentage of Ly6C⁺, CD62L⁺ and CD69⁺ cells within CD4⁺ and CD8⁺ gates. The cells exhibit activated phenotype characterized by downregulation of CD62L and upregulation of CD69. All cells were CD44 high within 2 weeks post-infection (data not shown), and remained Ly6C high during persistent *M. tuberculosis* infection.

5.3.4. Priming T cells specific for *M. tuberculosis* in the lung-draining lymph nodes

The priming of *M. tuberculosis*-specific IFN- γ T cell responses in lymph nodes of infected mice was determined by ELISPOT. T cells isolated from mediastinal lymph nodes were stimulated with infected wild-type dendritic cells that were incubated with either blocking anti-MHC Class I or anti-MHC Class II antibodies to estimate the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells, respectively. In addition, wild-type dendritic cells were pulsed with ESAT-6 protein, an antigen exclusively recognized by CD4⁺ T cells in C57BL/6 (H-2b) mice, to estimate the frequency of ESAT-6 specific CD4⁺ T cells. The priming of IFN- γ T cell responses peaked in the lung draining lymph nodes at 2 weeks post-infection (Figure 19A). Early in infection the priming of IFN- γ producing CD4⁺ T cells was prominent, and majority of these CD4⁺ T cells were also specific for ESAT-6 (Figure 19A and 19C). Surprisingly very few CD8⁺ T cells were primed to produce IFN- γ in acute infection (Figure 19B).

5.3.5. Dynamic changes in the frequency of *M. tuberculosis*-specific IFN- γ responses in the lungs

The majority of IFN- γ producing T cells in the lungs were CD4⁺ during acute infection (Figure 19A). Most of the IFN- γ CD4⁺ T cell response was directed against ESAT-6, which appeared to be immunodominant antigen in the acute infection (Figure 19C). However, as infection entered into the chronic stage the frequency of ESAT-6 specific, IFN- γ -producing CD4⁺ T cells decreased suggesting that other mycobacterial antigens were recognized by CD4⁺ T cells. Although very few IFN- γ producing CD8⁺ T cells were detected during acute infection, CD8⁺ T cells substantially contributed to the total IFN- γ production during chronic phase of

infection (Figure 19B). In fact, IFN- γ producing CD4⁺ and CD8⁺ T cells were present at equal frequencies during chronic infection (Figure 19A – 19B).

The most important finding of these experiments was that during the plateau stage when the number of bacteria and immune cells reached the steady state, the frequency of IFN- γ producing effector cells dynamically changed (Figure 19A and 19B). Both CD4⁺ and CD8⁺ T cell IFN- γ responses waxed and waned over the course of infection, suggesting bursts in the immune response. All the results were confirmed by intracellular cytokine staining (data not shown).

Since IFN- γ production is a key player in protection against tuberculosis, we next asked how many of IFN- γ producing T cells inside the infected lung were specific for *M. tuberculosis*. To address this question we directly compared the number of IFN- γ producing CD4⁺ and CD8⁺ T cells as estimated by intracellular cytokine staining and ELISPOT (Figure 19D). Non-specific stimulation of T cells with anti-CD3/CD28 antibodies will trigger IFN- γ production by all T cells that were primed to secrete IFN- γ . However, incubation of T cells with *M. tuberculosis*-infected dendritic cells will stimulate IFN- γ secretion only by *M. tuberculosis*-specific T cells. CD8⁺ T cells showed a strong positive correlation between the two assays indicating that most of CD8⁺ T cells that were primed to produce IFN- γ were specific for *M. tuberculosis* (Figure 19D). Although CD4⁺ T cells also showed a positive correlation, a significant portion of CD4⁺ T cells were activated bystanders (Figure 19D).

These results reflect a dynamic state of immune responses during the persistent *M. tuberculosis* infection, what was considered previously to be a steady balance between a pathogen and a host. In addition, these studies revealed significant kinetic and qualitative differences between *M. tuberculosis*-specific CD4⁺ and CD8⁺ T cells. Although priming of IFN- γ

producing CD8⁺ T cells was delayed, most of the CD8⁺ effector T cells were specific for *M. tuberculosis*. In contrast, IFN- γ producing CD4⁺ T cells were primed very early in infection; however, a significant proportion of CD4⁺ T cells were activated bystanders during persistent infection.

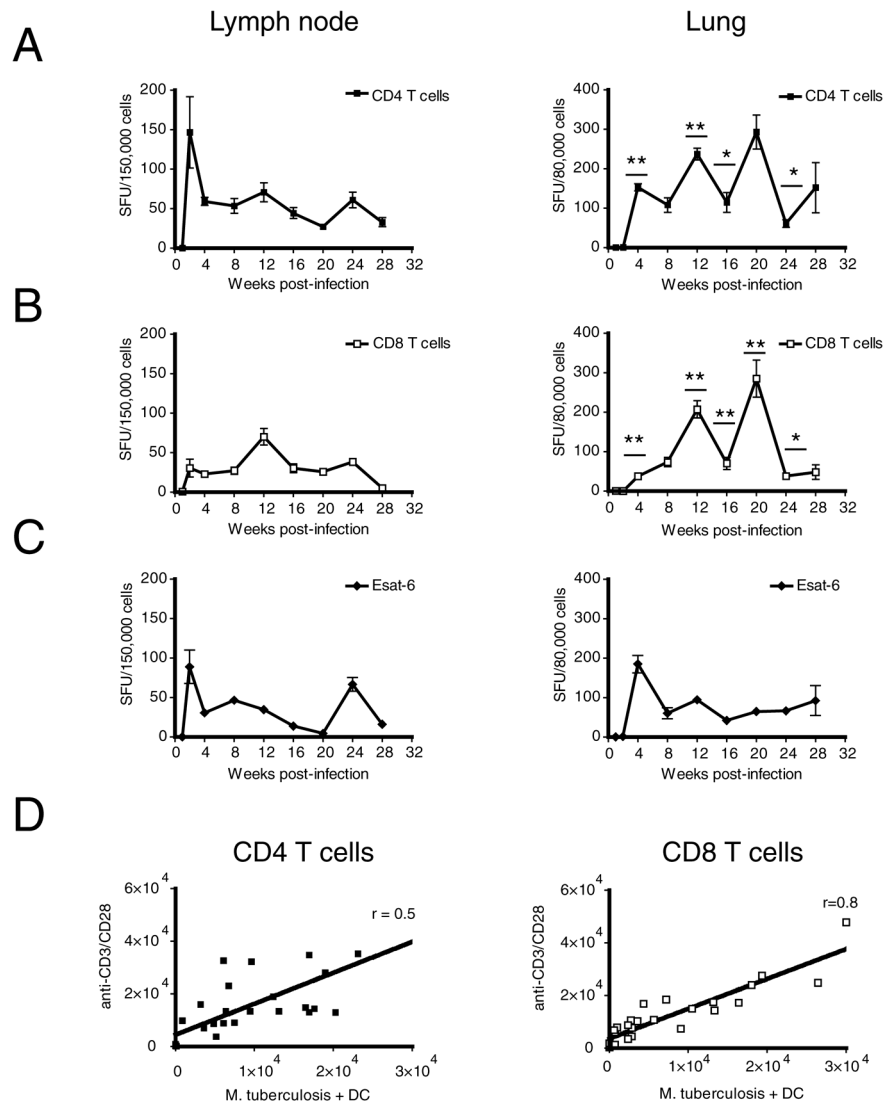


Figure 19 IFN- γ T cell responses in the lymph nodes and the lungs of *M. tuberculosis*-infected mice

(A and B) The graphs depict the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells, respectively, in the lymph nodes and the lungs of infected mice during a 7 month course of *M. tuberculosis* infection. The asterisks denote a statistically significant reduction in the frequency of IFN- γ producing T cells between the time points as determined by two-tail Student t-test. All the results were also confirmed with intracellular IFN- γ staining.

(C) The frequency of Esat-6 specific CD4⁺ T cell responses in the lymph nodes and the lungs.

(D) Comparison of the number of IFN- γ producing CD4⁺ and CD8⁺ T cells as estimated by ELISPOT (*M. tuberculosis*-specific responses) and intracellular cytokine staining (non-specific anti-CD3/CD28 antibody stimulation).

5.3.6. Differential regulation of CD8⁺ T cell effector functions in different phases of immune response against *M. tuberculosis*

The cytotoxic potential of CD8⁺ T cells during *M. tuberculosis* infection was analyzed using limiting dilution assay. Lung and lymph node cells of infected mice were plated in 2-fold serial dilutions and expanded during 2 week incubation with *M. tuberculosis*-infected antigen presenting cells. Following the expansion phase *in vitro*, the cytotoxicity of CD8⁺ T cells was tested against *M. tuberculosis*-infected, chromium labeled macrophages. The frequency of CTLp was determined by χ^2 minimization analysis. Flow cytometry analysis revealed that more than 80% of cells were CD8⁺ positive T cells after *in vitro* expansion (data not shown).

As shown in Figure 20A, CD8⁺ T cells were cytotoxic during acute infection; however, the frequency of CTLp declined as infection progressed into the chronic stage. The CD8⁺ T cells that were primed early in the lymph node had cytotoxic potential, and these cells were also cytotoxic in the lungs up to 4 weeks post-infection (Figure 20B). However, by 8 weeks post-infection, the CD8⁺ T cells in the lymph nodes and lungs had essentially no cytolytic potential (Figure 20B and 20C). As the frequency of CTLp decreased, the frequency of IFN- γ producing CD8⁺ T cells increased during the chronic infection, suggesting that there was a differential regulation of CD8⁺ T cell effector functions during different phases of immune response (Figure 20D).

To further evaluate the functional program of CD8⁺ T cells on a per cell basis during acute and persistent *M. tuberculosis* infection, we measured the percentage of perforin-producing cells within CD8⁺ IFN- γ positive and CD8⁺ IFN- γ negative populations using intracellular cytokine staining. *Ex vivo* isolated lung and lymph node cells were stimulated with anti-CD3/CD28 antibodies in the presence of monensin for 4 hours. Following incubation, cells were

fixed and permeablized, stained for perforin, IFN- γ and CD8 marker. During acute infection when CD8⁺ T cells exhibited cytotoxic potential, perforin detection was low within CD8⁺ T cell population (Figure 21B and 21C). The lack of perforin staining could be associated with *ex vivo* degranulation of CD8⁺ T cells during acute infection. Perforin expression was detected almost exclusively within IFN- γ negative CD8⁺ T cells during chronic infection, suggesting that these CD8⁺ T cells were clearly capable of synthesizing perforin at the time when their cytolytic activity was minimal (Figure 21B and 21C). It appears that these CD8⁺ T cells were not exhausted during persistent infection, but rather were not degranulating perforin in the face of constant exposure to *M. tuberculosis* antigens. We have confirmed perforin expression in the lungs of *M. tuberculosis*-infected mice using immunohistochemistry, and there was an increase in perforin staining in the chronic phase of infection (data not shown).

Since exposure of CD8⁺ T cells to low or high dose of antigens can determine the functional program of CD8⁺ T cells (238, 243-246), we performed direct comparison between bacterial load as determined by number of colony forming units and frequency of CTLp in the lungs of infected mice. We did not find a direct correlation between the total number of live replicating bacteria and cytotoxic activity of CD8⁺ T cells, suggesting that the functional program of CD8⁺ T cells may not be governed by the number of viable mycobacteria. However, mycobacteria that are killed by the immune response or even by drugs are not quickly cleared (247, 248), so cumulative antigen load may contribute to the change in CD8⁺ T cell function.

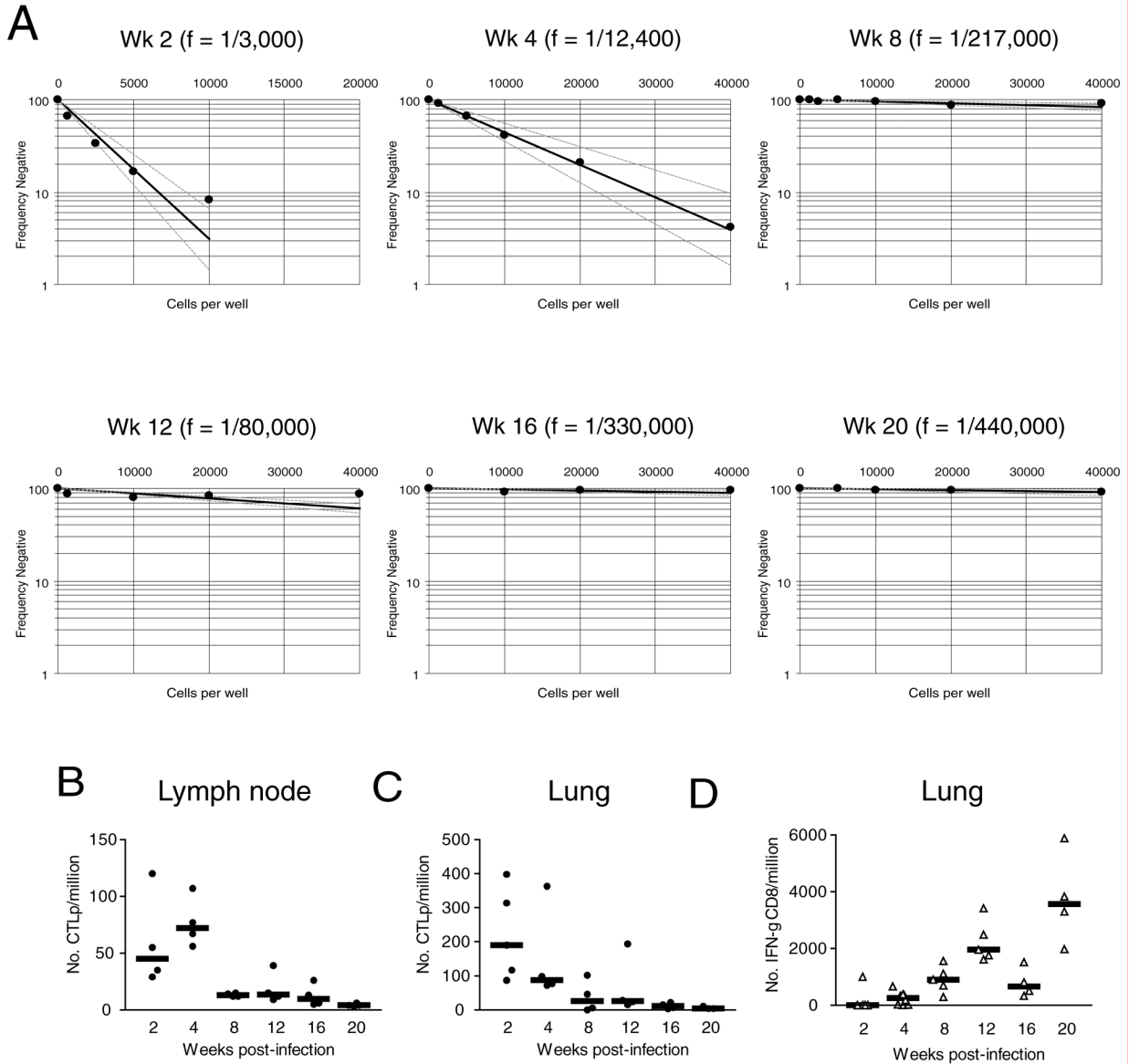


Figure 20 The frequency of cytotoxic $CD8^+$ T cells decreases as the frequency of $IFN-\gamma$ producing $CD8^+$ T cells increases during chronic *M. tuberculosis* infection

(A) The frequency of CTLp was estimated by limiting dilution assay using χ^2 minimization analysis as described in Materials and Methods.

(B and D) The median number of CTL precursors per million lymph node and lung cells. Each filled circle represents an individual mouse. The line represents the median number of CTLp for all mice in each group (4 – 5 mice).

(D) The median number of $IFN-\gamma$ producing $CD8^+$ T cells per million lung cells.

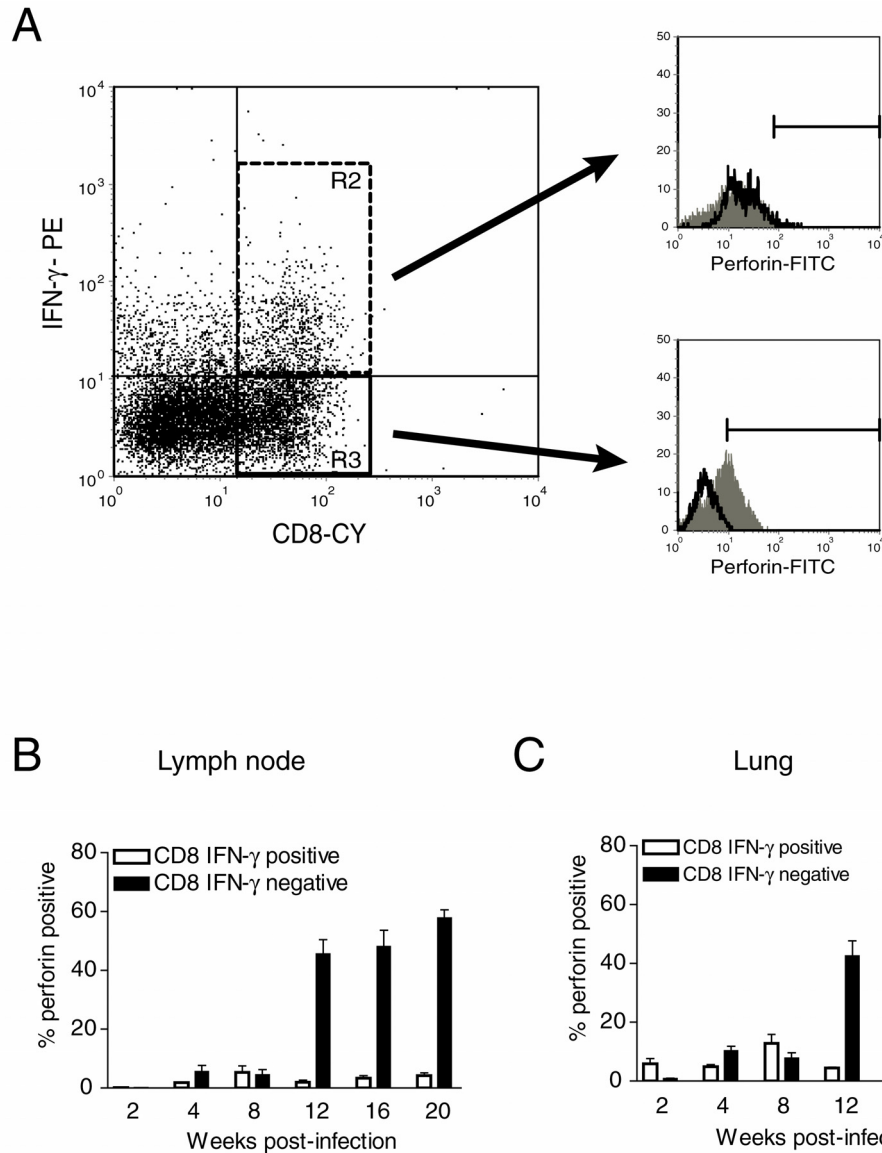


Figure 21 Perforin staining was limited only to IFN- γ negative CD8⁺ T cell population

There was minimal cytotoxic activity despite significant perforin expression during chronic infection. To determine the percentage of perforin positive cells within IFN- γ positive and IFN- γ negative CD8⁺ T cells, we adapted the method from Slifka *et al.* (242).

(A) The populations were gated as shown in the panel A.

(B and C) Single cell suspensions from the lymph nodes (B) and the lungs (C) were prepared at designated time points, and stimulated *ex vivo* with anti-CD3 and anti-CD28 antibodies in the presence of monensin for 4 hours at 37°C. Following stimulation cells were fixed-permeabilized and stained with anti-perforin, anti-IFN- γ and anti-CD8⁺ antibodies as described in Material and Methods. The graphs in B and C show the percentage of perforin expressing cells within CD8⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁻ cells.

5.4. Discussion

The primary aim of this study was to determine how chronic exposure to high antigen dose influences the functionality of *M. tuberculosis* specific T cell responses. In contrast to models of chronic viral infection, we found no evidence of paralysis or permanent loss of effector functions during chronic *M. tuberculosis* infection. The main findings of our study indicate that both CD4⁺ and CD8⁺ T cells underwent two bursts of replication during a 7 month course of *M. tuberculosis* infection. Although CD4⁺ T cells were the predominant source of IFN- γ during acute infection, both CD4⁺ and CD8⁺ T cells equally contributed to IFN- γ production during chronic infection. There were dynamic changes in the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells during the stable chronic phase of infection. Surprisingly, CD8⁺ T cells exhibited differential effector functions at different phases of infection; they were mainly cytotoxic T lymphocytes during acute infection, but switched to IFN- γ production during chronic infection. The lack of cytotoxic activity was not associated with the loss of perforin staining, suggesting that CD8⁺ T cells were restrained from employing their cytotoxic effector functions.

In viral models of chronic infections it has been shown that T cells are incapable of mounting adequate effector functions to facilitate viral clearance, despite being persistently activated. CD8⁺ T cells may completely lose their cytotoxic capability, often marked by the absence of perforin staining, or may sequentially lose the ability to elaborate key cytokines, such as IL-2, TNF- α and IFN- γ (229-237). Detrimental effects to the host immune system by chronic exposure to high antigen doses are not limited to CD8⁺ T cells. CD4⁺ T cell responses also demonstrate marked dysregulation of their effector functions. CD4⁺ T cells lose their ability to produce IL-2 and IFN- γ and to provide help for CD8⁺ T cell function (233, 234). In addition, following exposure to persistently elevated antigen doses, T cells enter a state of replicative

senescence, in which T cells remain metabolically active but fail to proliferate (237). Our data demonstrate that the interaction of *M. tuberculosis* with the immune system, in which a chronic infection is established, is very different than the viral systems that have been studied to date.

A common feature of persistent viral infections is that following exposure to high antigen doses, virus-specific T cells undergo accelerated and excessive T cell turnover resulting in replicative senescence. Our results using staining for BrdU incorporation and annexin V/7AAD expression on the same lung cells suggest that over the 7 months of *M. tuberculosis* infection, CD4⁺ and CD8⁺ T cells did not lose the capacity to proliferate. Both cell populations underwent two successive bursts of replication in the lungs. The level of apoptosis occurring within the lung paralleled the proliferation kinetics, whereby increased apoptosis of the cells was accompanied by increased proliferation. During the shift from acute to chronic infection, once bacterial numbers were stabilized, increased apoptosis and reduced proliferation contributed to the contraction phase of the T cell responses. However, the stabilization of T cell numbers during chronic infection was accompanied by steady levels of apoptosis. This suggests that infiltration of new effector T cells into the lungs is likely to be ongoing during chronic infection.

Flow cytometric analysis demonstrated that both CD4⁺ and CD8⁺ T cell populations remained in an activated state, characterized by downregulation of CD62L and high expression of Ly6C and CD69.

Priming of *M. tuberculosis*-specific IFN- γ responses peaked at 2 weeks post-infection. IFN- γ specific ELISPOT and intracellular cytokine staining showed that CD4⁺ T cells were responsible for the majority of IFN- γ production during acute infection, as suggested previously (132). The priming and the presence of IFN- γ producing CD8⁺ T cells in the infected lungs was negligible until 8 weeks post-infection. Most of IFN- γ producing CD4⁺ T cells were specific for

ESAT-6, which appeared to be the dominant antigen in acute infection (132). However, during chronic infection the frequency of ESAT-6 specific CD4⁺ T cells in the lungs decreased, indicating that other mycobacterial antigens were being recognized.

Although CD8⁺ T cells contributed minimally to IFN- γ production during acute infection, the frequency of IFN- γ producing CD8⁺ T cells was equivalent to IFN- γ producing CD4⁺ T cells during chronic infection. A surprising result was that the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells dynamically waxed and waned during persistent *M. tuberculosis* infection. These results could be explained by two possible scenarios: (i) during chronic infection mycobacteria are likely mostly quiescent; however, they may transiently undergo bursts of replication. These changes in bacterial numbers are sensed by the host immune system, which responds in turn by rapidly increasing the numbers of IFN- γ producing T cells until bacterial growth is brought under control. In this manner, bacterial growth will be strictly controlled, with minimal immunopathology that otherwise would be induced by persistently elevated levels of inflammatory cytokines, such as IFN- γ . This explanation suggests a dynamic equilibrium achieved between the pathogen and the host's immune system that persists throughout the course of infection. (ii) Alternatively, T cells may be rendered transiently anergic and unable to respond to constant antigenic stimulation, which would be characterized by drops in the frequency of IFN- γ producing T cells. The regular periodicity of the peaks and troughs in the frequency of IFN- γ producing T cells suggests that a certain time interval is required for T cells to renew their IFN- γ producing capacity. A detailed investigation of TCR signaling at different time points during chronic infection will resolve whether transient anergy in T cell activation exists due to persistent exposure to mycobacterial antigens. Nevertheless, the T cell IFN- γ production is not

permanently silenced by high antigen doses, suggesting that the effects of persistent mycobacterial antigenic stimulation on T cell functionality are transient.

Our findings point to kinetic and qualitative differences in *M. tuberculosis*-specific CD4⁺ and CD8⁺ T cell responses. Although CD4⁺ T cells outnumbered CD8⁺ T cells in the lungs throughout infection, a greater percentage of CD8⁺ T cells was activated, and nearly all of them were specific for *M. tuberculosis*, in contrast to CD4⁺ T cells. While CD4⁺ T cells responded early to *M. tuberculosis* challenge, priming of IFN- γ producing CD8⁺ T cells occurred after chronic infection was established.

Longitudinal analysis of CD8⁺ T cell effector functions revealed that there was a switch in the functional program of CD8⁺ T cells during the course of infection. Early in infection, CD8⁺ T cells were predominantly cytotoxic, but during chronic infection CD8⁺ T cells switched to cytokine production. The lack of cytotoxicity by CD8⁺ T cells was not accompanied by the expected loss of perforin staining, as most of IFN- γ negative CD8⁺ T cells continued to express perforin during chronic infection.

The dual staining for IFN- γ and perforin within the CD8⁺ T cell population revealed two important findings. First, the split between cytokine production and cytotoxic functions was clearly evident on per cell basis, as perforin staining segregated to only the IFN- γ negative T cells. Second, although CD8⁺ T cells exhibited minimal cytotoxicity in chronic infection, their ability to synthesize perforin remained unimpaired. In fact, when measurable cytotoxic function was high, it was difficult to detect intracellular perforin staining, suggesting that perforin was released from the cells efficiently. Therefore, it appears that the lack of CTL activity was not a result of CD8⁺ T cell exhaustion but rather a consequence of factors that dictate the functional

program of CD8⁺ T cells during persistent *M. tuberculosis* infection. The clearest candidate accounting for these observations is antigen dose.

Several studies reported that the level of TCR occupancy can determine the development fate of CD8⁺ T cells *i.e.* whether CD8⁺ T cells will be cytotoxic or produce cytokines (119, 243-246). At low antigen doses, an immature immunologic synapse is formed, which is sufficient to trigger cytotoxicity, but not cytokine production or proliferation (245, 246). Conversely, at high levels of TCR occupancy, a mature immunological synapse is formed that due to sustained and heightened levels of Ca²⁺ intracellular signaling will lead to preferential cytokine production by CD8⁺ T cells (245, 246). Most of these studies were performed on CD8⁺ T cells clones and by pulsing antigen presenting cells with different peptide concentrations.

Very few reports exist on the behavior of primary CD8⁺ T cells during *in vivo* infection. Betts *et al.* showed that in primary human HIV- and CMV- specific CD8⁺ T cells, antigen dose was the sole determinant of the cytokine versus cytotoxic nature of virus specific CD8⁺ T cell responses (238). Using *M. tuberculosis*-specific human CD8⁺ T cell clones, Lewinsohn *et al.* demonstrated that CD8⁺ T cells preferentially lysed heavily infected cells (249). These CD8⁺ T cell clones were generated from the peripheral blood of latently infected individuals in which it is believed the presence of *M. tuberculosis* antigen is minimal or undetectable. Results from several epidemiological studies indicate that the cytotoxic activity of CD8⁺ T cells was dependent on the clinical state of TB patients. De la Barrera *et al.* reported that while cytotoxic activity was readily detectable in healthy PPD⁺ individuals, the cytotoxic potential of CD4⁺ and CD8⁺ T cells was significantly diminished in patients with active tuberculosis (250). Therefore, cell lines and clones from healthy PPD⁺ individuals may not represent the physiological state of

CD8⁺ T cells in patients with active or chronic tuberculosis where CD8⁺ T cells may be exposed to high antigen dose over long periods of time.

The concept of antigen dose shaping the developmental fate of CD8⁺ effector functions is particularly attractive in our tuberculosis model. During acute infection very few macrophages are infected, and the number of mycobacteria is low; hence, cytotoxicity may be the preferential mode of action for CD8⁺ T cells early in infection. During chronic infection when a large number of macrophages are infected, the antigen dose is higher, and CD8⁺ T cells switch to cytokine production. In this manner, the immunopathologic effects of persistent cytotoxic activity during long term *M. tuberculosis* infection may be minimized.

We were unable to correlate CD8⁺ CTLp frequency with the total number of viable bacteria in the lungs. However, determination of CFUs is a crude estimate of antigenic load that CD8⁺ T cells may be exposed to *in vivo*. It measures only the number of replication-competent mycobacteria. It is very difficult to estimate the epitope density on the surface of infected macrophages. Russell *et al.* reported that mycobacterial antigens, particularly lipids and glycolipids, traffic dynamically to the cell surface of infected cells (251-253). Thus, the physiological relevance of antigen dose on effector CD8⁺ T cell functions in *M. tuberculosis* infection remains to be resolved.

An alternative explanation for the differential effector functions of CD8⁺ T cells was proposed by Sad *et al.* who suggest that initially CD8⁺ T cells kill the antigen presenting cells that they encounter, but repeated stimulation of CD8⁺ T cells with higher numbers of APCs eventually leads to cytokine production (243). It is tempting to speculate in our model that at first *M. tuberculosis*-specific CD8⁺ T cells will kill infected macrophages and following repeated exposure to infected macrophages, CD8⁺ T cells will preferentially produce inflammatory

cytokines. This switch will activate RNI and ROS pathways in infected macrophages leading to the destruction of intracellular bacteria, thereby minimizing immunopathology that would otherwise be invoked by extensive cytotoxic activity during infection.

Overall, our study indicates that the long-term control of *M. tuberculosis* is achieved through dynamic immune responses that persist even during the chronic phase of infection, including waxing and waning of cytokine production and shifts in effector phenotypes. Comparison of antigen specific and non-specific stimulation revealed that while most of CD8⁺ T cells were antigen-specific, a significant portion of CD4⁺ T cells were activated bystanders. Although IFN- γ production by CD8⁺ T cells was delayed when compared to CD4⁺ T cells CD8⁺ T cells responded early by preferentially employing their cytotoxic effector mechanisms. Cytokine production by CD8⁺ T cells occurred after transition to the chronic stage of infection. The lack of cytotoxic activity during chronic infection was not accompanied by the expected loss of perforin staining suggesting that certain components of immune system may regulate the effector function of CD8⁺ T cells. We showed previously that CD4⁺ T cells are required for cytotoxic functions of CD8⁺ T cells, suggesting that the immune response to *M. tuberculosis* is cross-regulatory. Understanding of the *in vivo* mechanisms that regulate the effector functions of T cells in the face of persistent *M. tuberculosis* infection is pivotal for the improved design of anti-tuberculosis vaccine strategies.

6. CHAPTER 3 INDUCTION OF *MYCOBACTERIUM TUBERCULOSIS*-SPECIFIC PRIMARY AND SECONDARY T CELL RESPONSES IN IL-15^{-/-} MICE

6.1. Introduction

IL-15 exerts its biological effects on multiple cell types as a result of wide distribution of its receptor. IL-15^{-/-} and IL-15R α ^{-/-} mice exhibit lymphopenia due to marked reductions of thymic and peripheral NK, NK T cells, and TCR $\gamma\delta$ intraepithelial lymphocytes (IEL) (254, 255). Furthermore, in the absence of IL-15 signaling the population of CD8⁺ T cells with memory phenotype (CD8⁺ CD44^{hi}) was significantly diminished (254, 255).

It was initially reported that proliferation of memory CD8⁺ T cells was considerably enhanced after injection of LPS and Poly I:C into mice as a result of increased type I IFN production (256). Zhang *et al.* identified IFN I to be a potent inducer of IL-15, which strongly and selectively stimulated proliferation of memory CD8⁺ T cells both *in vivo* and *in vitro* (256). IL-2/IL-15R β (a common receptor subunit for these two cytokines) is highly expressed on memory CD8⁺ CD44^{hi} T cells while expression of this receptor subunit is low on naïve CD8⁺ T cells and memory CD4⁺ T cells (257). Treatment of cells with the blocking anti-IL-2/IL-15R β antibody, but not with anti-IL-2 antibody, markedly reduced the numbers of proliferating memory CD8⁺ T cells (257). Additional evidence for the role of IL-15 in regulating homeostasis of memory CD8⁺ T cells came from IL15 transgenic mice, which have markedly increased numbers of memory CD8⁺ T cells (258, 259).

IL-15 signaling is also essential for antigen-presenting functions of dendritic cells. Stimulation of dendritic cells with IL-15 resulted in up-regulation of co-stimulatory molecules, increased production of IFN- γ and enhanced capacity of dendritic cells to stimulate proliferation

of CD8⁺ T cells (260). Dendritic cells and macrophages from γ_c -/-, IL-2/IL-15R β -/-, IL-15-/-, but not from IL-2-/-, mice showed impaired production of IL-12, IFN- γ and NO and reduced levels of antigen-presenting and co-stimulatory molecules (261). IL-15 stimulated human monocytes to produce IL-12 upon contact with CD4⁺ T cells via CD40/CD40L interaction, and thus contributed to IL-12-mediated induction of IFN- γ secretion by CD4⁺ T cells (262).

Due to pleotropic effects of IL-15 on multiple cell types of innate immunity and CD8⁺ T cell compartment, it was not surprising that IL15-/- and IL-15R α -/- mice exhibited compromised host defense responses against viral and bacterial pathogens. Although generation of lymphocytic choriomeningitis virus (LCMV)-specific effector CD8⁺ T cell responses was unimpaired in IL-15 deficient mice (263), the absence of IL-15 had a profound effect on the maintenance of LCMV-specific memory CD8⁺ T cell responses (263). Generation of primary and memory CD8⁺ T cell responses against vesicular stomatitis virus (VSV) was dependent on IL-15 signaling as longitudinal analysis revealed a slow decline in virus specific memory CD8⁺ T cells in IL-15-/- and IL-15R α -/- mice (264).

Treatment of *Toxoplasma gondii* immune mice with soluble IL-15R α markedly reduced the ability of treated mice to control infection (265). CD8⁺ T cell responses in sIL-15R α administered mice demonstrated reduced IFN- γ production, cytolytic activity and replicative capacity in response to *T. gondii* infection (265). In contrast, treatment of *T. gondii*-infected mice with IL-15 augmented and prolonged the duration of CD8⁺ T cell mediated immunity against *T. gondii* infection (266, 267). Similarly, IL-15 transgenic mice had significantly increased numbers of memory CD8⁺ T cells and conferred higher level of resistance against *Listeria* and *Salmonella* infections (258, 259, 268). Collectively, these studies demonstrate the importance of IL-15 in protective immunity against viral, parasitic and bacterial infections.

Several lines of evidence indicate that IL-15 may play a role in protective immunity against mycobacterial infections. Detection of IL-15 mRNA and IL-15 protein in the skin lesions of *Mycobacterium leprae* infected patients was previously reported (269, 270). While Julien *et al.* detected increased levels of IL-15 mRNA in patients with resistant tuberculoid lesions versus patients with susceptible lepromatous lesions, immunohistochemical staining of skin biopsies revealed similar levels of IL-15 protein in both forms of the disease (270). Infection of murine macrophages with *M. bovis* BCG and *M. tuberculosis* H37Ra in the presence of IFN- γ resulted in increased IL-15 mRNA expression (271). *In vitro* infection of monocyte-derived macrophages isolated from healthy blood donors with *M. tuberculosis* induced significant IL-15 protein secretion (270). Immunohistochemical analysis revealed that significantly higher number of alveolar macrophages isolated from bronchoalveolar lavage of tuberculosis patients produced IL-15 when compared to healthy subjects suggesting that IL-15 expression is induced following *M. tuberculosis* infection in humans (272). In murine studies, IL-15 transgenic mice exhibited increased resistance to *M. bovis* BCG infection, which in part could be attributed to increased numbers of NK cells and augmented IFN- γ production by CD8⁺ T cells (273). Furthermore, IL-15 improved the efficacy of *M. bovis* BCG vaccine in conferring protection against virulent *M. tuberculosis* H37Rv challenge. BCG-vaccinated IL-15 transgenic mice had significantly lower bacterial burden when compared with BCG-vaccinated wild-type mice (274). The increased protective effect observed in IL-15 transgenic BCG vaccinated mice was accompanied by enhanced IFN- γ CD8⁺ T cell responses (274). IL-15 administration also protected susceptible BALB/c mice against virulent *M. tuberculosis* infection when given as a treatment at 3 weeks post-infection (275).

Although these studies collectively show that IL-15 is expressed following mycobacterial infections, and that IL-15 exogenous administration can enhance protective immunity against *M. tuberculosis* infection, there is no information about the functionality of *M. tuberculosis*-specific primary and secondary T cell responses in the absence of IL-15. In this study, we evaluated the quality of CD4⁺ and CD8⁺ effector and memory T cell responses against *M. tuberculosis* infection in IL-15^{-/-} mice. Our data indicate that IL-15^{-/-} mice exhibited slightly higher bacterial burden in the lungs during chronic *M. tuberculosis* infection, which was accompanied by an increase in the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cell responses. The baseline level of CD8⁺ T cells in uninfected IL-15^{-/-} mice was ~ 30 - 50% lower than in uninfected wild-type mice. The remaining CD8⁺ T cells from IL-15^{-/-} mice did not show increased apoptosis or a defect in their replicative capacity in response to *M. tuberculosis* infection. There were significantly fewer CD4⁺ and CD8⁺ T cells in the lungs of immune IL-15^{-/-} and wild-type mice early after secondary challenge. Similar percentages of proliferating and IFN- γ producing memory CD4⁺ and CD8⁺ T cells were detected in IL-15^{-/-} and wild-type mice after *M. tuberculosis* secondary infection. These findings suggest that generation of *M. tuberculosis*-specific effector CD4⁺ and CD8⁺ T cells responses is unimpaired in IL-15^{-/-} mice.

6.2. Materials and Methods

6.2.1. Mice and infections

IL-15^{-/-} and C57BL/6 wild-type mice were purchased from Taconic (Germantown, NY) and Charles River Laboratories (Wilmington, MA), respectively. All mice were kept under specific pathogen-free conditions in a Biosafety Level 3 facility. Animal protocols used in this study were approved by the University Institutional Animal Care and Use Committee. For the primary infection and secondary challenge mice were infected with a low dose of *M. tuberculosis* (Erdman strain, Trudeau Institute, Saranac Lake, NY) at 5x10⁵/ml using a nose-exposure only aerosolizer unit (Intox Inc., Moriarty, NM). The dose received was estimated by plating whole lung homogenates of two mice 24 hours following aerosol infection (~30 CFU/mouse).

6.2.2. Memory IL-15^{-/-} mice

Mice were infected with a low dose of *M. tuberculosis* (~30 CFU/mouse) via aerosol route. From 4 weeks post-infection, mice were treated with isoniazid (0.1 g/liter) and pyrazinamide (15 g/liter) in drinking water two times a week for 8 weeks to clear the infection. As IL-15^{-/-} mice were unable to clear *M. tuberculosis* with this antibiotic therapy, the treatment was changed to isoniazid (0.1g/liter) and rifampicin (0.15 g/l) for additional 16 weeks. At the end of antibiotic treatment mice were sacrificed and several organ homogenates (lung and spleen) were plated on 7H10 plates (Difco) to confirm the absence of viable mycobacteria. Mice were challenged with a low dose of *M. tuberculosis* via aerosol route and the quality of CD8⁺ memory T cell responses was investigated after secondary *M. tuberculosis* infection.

6.2.3. CFU determination

Lung homogenates were serially diluted in PBS/0.05% Tween-80 and plated on 7H10 agar plates (Difco). Plates were incubated at 37°C, 5% CO₂ for 21 days prior to counting colonies.

6.2.4. Bone marrow derived macrophages and dendritic cell cultures

In *ex vivo* stimulation assays, such as ELISPOT and limiting dilution analysis, bone marrow derived dendritic cells were cultured in the presence of GM-CSF supernatant at 1:200 dilution (a generous gift from Dr. Binfeng Lu, University of Pittsburgh) and 20 ng/ml of IL-4 (PeproTech Inc, Rocky Hill, NJ) and macrophages were cultured in the presence of L cell supernatant as a source of CSF-1 using standard procedure described previously (146).

6.2.5. Flow cytometry

Lung single cell suspensions were stained as described previously (146). Cells were stained with anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7), and anti-CD69 (clone H1.2F3) fluorescently conjugated antibodies. All antibodies were purchased from BD Pharmingen (San Diego, CA) and used at 0.2 µg/ml concentration. Cells were collected on a FACSCaliber (Beckon Dickinson, San Jose, CA) and analyzed by CellQuest (Becton Dickinson) or FlowJo (Tree Star Inc, San Carlos, CA) software.

6.2.6. Proliferation of T cells in the lungs of infected mice

Sixteen hours prior to each experimental time point, mice were injected intraperitoneally with saline containing 1 mg of 5-bromo-2'-deoxyuridine [BrdU] (Sigma-Aldrich, St. Louis,

MO). Lung cells were stained for cell surface markers CD4 and CD8 at room temperature for 20 minutes prior to a fixation step with 4% paraformaldehyde [PFA] (200 µl/tube) on ice for 20 minutes. Cells were washed with tissue culture phosphate buffered saline [PBS], centrifuged at 470 g, and cell pellets were suspended in ice-cold 0.15 M NaCl (100 µl/tube), followed immediately by drop-wise fixation with ice-cold 95% ethanol (200 µl/tube) on ice for 30 minutes. Following a PBS wash, cells were permeabilized and fixed with 200 µl/tube of 0.4% saponin and 2% PFA for 1 hour at room temperature. Cells were washed with PBS, and suspended in 200 µl/tube of 0.15 M NaCl, 4.2 mM MgCl₂ (pH 5) containing 250 U/ml of DNase I (Roche, Indianapolis, IN) for 30 minutes in 37°C water bath. Cells were washed with PBS, and incubated with anti-BrdU antibody or the respective isotype control (BD Pharmingen, FITC-conjugated antibody set) diluted at 1:3 in 0.5% Tween-20 and 0.5% BSA (50 µl/tube). Following 30 minute incubation at room temperature, cells were washed with PBS, and fixed with 4% PFA prior to acquisition on the flow cytometer.

6.2.7. Apoptosis staining

Lung cells were stained for the expression of CD4 and CD8 molecules for 20 minutes at room temperature. The amount of apoptosis was determined by staining lung cells with Annexin V-FITC and 7-AAD reagents (BD Pharmingen) according to manufacturer's instructions. Briefly, cells were suspended in 100 µl/tube of 1x Binding buffer, and incubated with 5 µl/tube of Annexin V-FITC and 5 µl/tube of 7-AAD for 15 minutes in the dark at room temperature. Cells were washed with 2 ml/tube of 1x Binding buffer to remove any unbound Annexin V-FITC and 7-AAD, and fixed with 4% PFA in 1x Binding buffer. Samples were analyzed within 30 minutes.

6.2.8. IFN- γ production

Cytokine production by CD8⁺ T cells isolated from the lungs of *M. tuberculosis* infected mice was evaluated by ELISPOT as described previously (240, 241). Briefly, lung and lymph node cells were plated in anti-IFN- γ antibody (BD Pharmingen, clone R4-6A2) coated plates (MAIPS4510, Millipore Corp, Bedford, MA) at 80,000 cells/well and 150,000 cell/well, respectively. Cells were incubated in duplicate wells with media, ConA (10 μ g/ml; Sigma-Aldrich), uninfected and *M. tuberculosis*-infected dendritic cells (MOI 3; overnight) to estimate the number of total number of IFN- γ producing T cell, and *M. tuberculosis*-infected dendritic cells incubated with the blocking anti-MHC Class I (BD Pharmingen, clone 8F12) or anti-MHC Class II (BD Pharmingen, clone M5/114.15.2) antibodies at 10 μ g/ml to estimate the number of IFN- γ producing CD4⁺ and CD8⁺ T cells, respectively. All dendritic cells were added to lung and lymph nodes cells at 1:2 ratio and the cultures were supplemented with IL-2 (PeproTech) at final concentration of 20 U/ml.

Following 40 hour incubation, the IFN- γ producing T cells were visualized after stepwise incubation of plates with biotinylated anti-IFN- γ antibody (BD Pharmingen, clone XMG 1.2), streptavidin-conjugated enzyme (PK-6100, Vector Laboratories) and AEC substrate (SK-4200, Vector Laboratories). The spot forming units (SFU) were enumerated using ELISpot reader (Cellular Technology Ltd, Cleveland, OH).

6.2.9. Limiting dilution analysis

The cytotoxic potential of CD8⁺ T cells was estimated using limiting dilution analysis (LDA) (240, 241). Effector cells were derived from the lungs and lung draining lymph nodes of

M. tuberculosis-infected mice at designated time points. Freshly isolated cells were plated in 2-fold serial dilutions starting from 40,000 cells/well to 1250 cells/well in V-bottom 96-well plates (24 replicates/input number) supplemented with IL-2 at 20 U/ml. Lung and lymph node cells were incubated with *M. tuberculosis*-infected dendritic cells (500 DC/well) for 7 days. Following incubation, 100 μ l of spent media was removed from each well, and lung cells were cultured for another round of stimulation with *M. tuberculosis*-infected macrophages (1000 M Φ /well) and IL-2 (20 U/ml) to allow for expansion of CTL precursors. Flow cytometry analysis revealed that 75-95% of cells were CD8⁺ T cells after 2 weeks of stimulation.

Cytotoxicity was determined in each well by a standard ⁵¹Chromium release assay with *M. tuberculosis*-infected macrophages as targets. *M. tuberculosis*-infected macrophages were labeled with ⁵¹Cr (100 μ l of ⁵¹Cr per 3x10⁶ macrophages) for 1 hour at 37°C, and added to lung and lymph node T cell cultures at 4000 cells/well. Following 4 hour incubation, 100 μ l of supernatant was collected (Skatron SCS System; Skatron, Sterling, VA) and radioactivity was quantified using a gamma counter. Positive wells were defined as being greater than mean + 3 SD of spontaneous target cell release. Frequency of CTLp was determined using zero-order Poisson equation ($\ln Y = -Fx + \ln A$; where x = the number of effector cells/well; Y = % negative wells; A = the y-axis intercept; F = CTLp frequency defined by the negative slope of the line). All calculations were performed using a software program fitted to the equation by χ^2 minimization analysis (a generous gift from Dr. Carolyn A. Keever-Taylor, Medical College of Wisconsin).

6.2.10. Statistics

Statistically significant differences in the numbers of effector T cells between the two time points were determined using unpaired, two-sided, Student t-test. The p-value of < 0.05 was defined as being significant.

6.3. Results

6.3.1. IL15^{-/-} mice are not susceptible to *M. tuberculosis* infection

IL15^{-/-} and wild-type mice were infected with a low dose of *M. tuberculosis* via aerosol route. At designated time points, lung, spleen and lymph node homogenates were plated to determine the number of colony forming units (CFU). IL15^{-/-} mice were equally capable of controlling *M. tuberculosis* infection in the lungs during acute infection; however, IL-15^{-/-} mice harbored a slightly higher bacterial burden during chronic infection (reproducible in three separate experiments) (Figure 22). There were no significant differences in bacterial numbers in the spleen and lymph nodes of IL-15^{-/-} and wild-type mice at any time point (Figure 22).

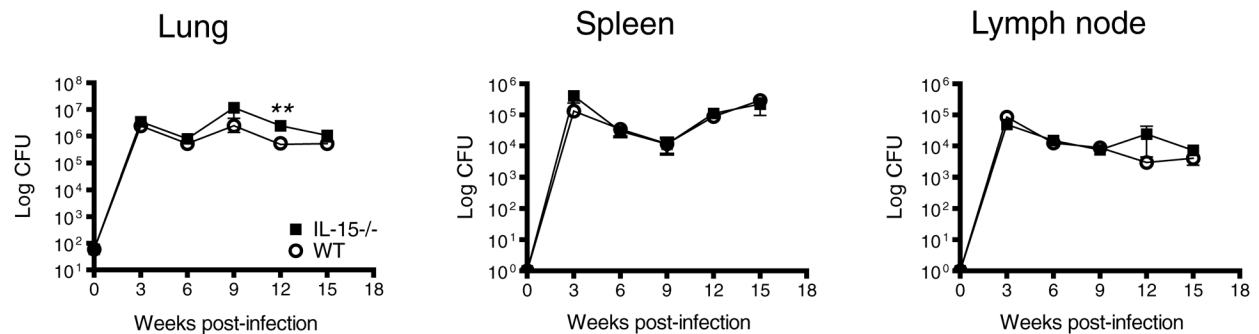


Figure 22 IL-15^{-/-} mice control low-dose *M. tuberculosis* infection

IL-15^{-/-} and wild-type mice were infected with a low dose of *M. tuberculosis* via aerosol route. At designated time points, serial dilutions of lung, spleen and lymph nodes were plated on 7H10 plates and the colony forming units (CFU) were enumerated after 21 days of incubation. The results represent the mean \pm SEM of four mice per group, and the experiments were replicated three times with similar results.

6.3.2. CD4⁺ and CD8⁺ T cell responses during primary *M. tuberculosis* infection

Overall numbers of cells infiltrating the lung and within the lymph nodes were lower in IL-15^{-/-} mice; however, differences were not always statistically significant (Figure 23A). The percentage of CD4⁺ and CD8⁺ T cells and their activation status were determined by flow cytometry after staining lung and lymph node cell suspensions with anti-CD4, anti-CD8 and anti-CD69 antibodies. IL15^{-/-} mice had a similar percentage of CD4⁺ T cells in the lungs and lymph nodes when compared to wild-type mice (Figure 23B). In contrast, uninfected IL-15^{-/-} mice had 50% fewer CD8⁺ T cells in the lungs and ~ 70% fewer CD8⁺ T cells in the lymph nodes when compared to uninfected wild-type mice (Figure 23C). A 2-fold difference in the percentages of CD8⁺ T cells was maintained throughout the infection. There was a similar percentage of activated CD4⁺ T cells, while significantly more CD8⁺ T cells were activated in IL-15^{-/-} mice during chronic infection (Figure 23D).

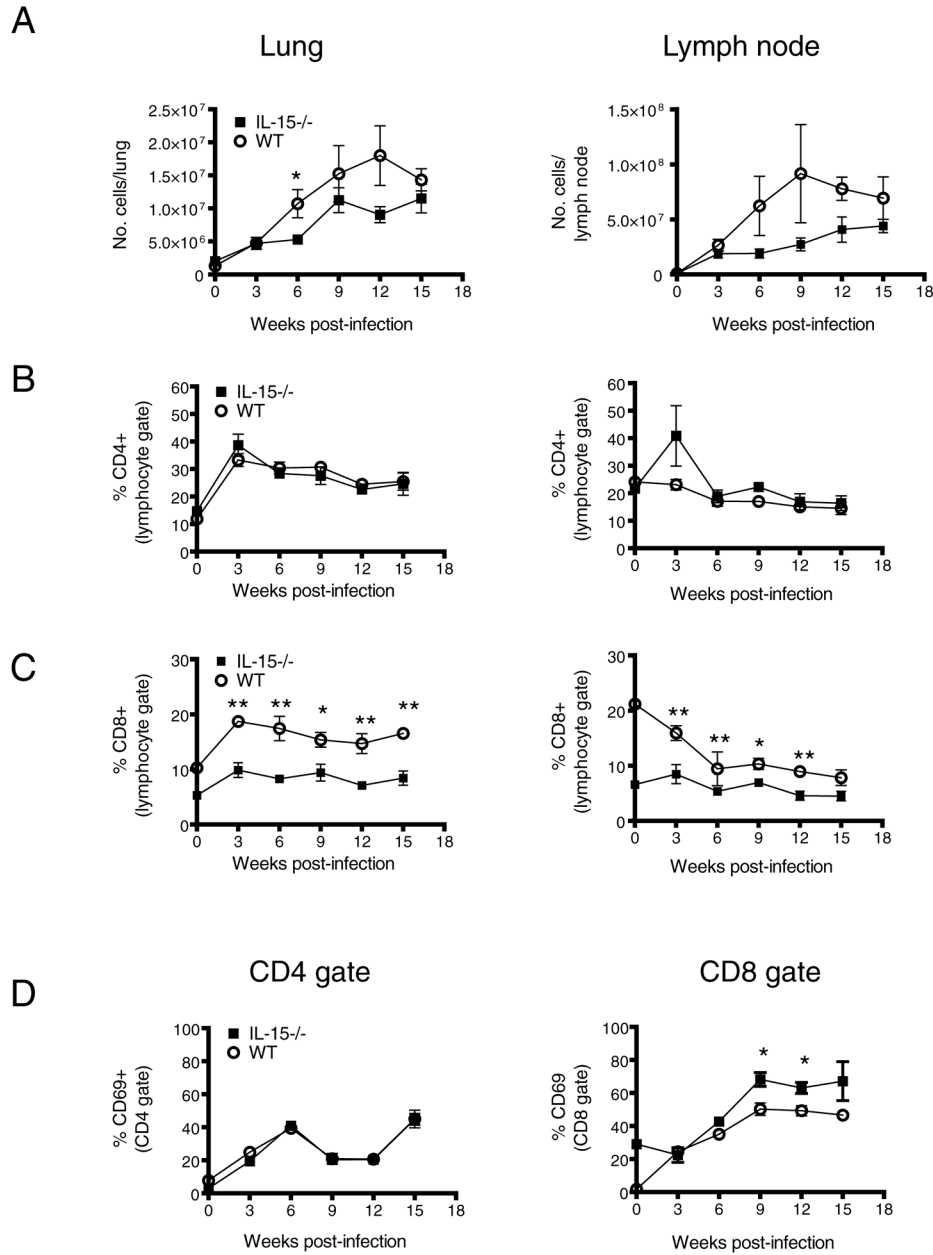


Figure 23 Phenotypical characterization of immune cells infiltrating the lungs of IL-15^{-/-} and wild-type mice

(A) Overall numbers of live cells inside the lungs and lymph nodes of IL-15^{-/-} and wild-type mice were determined by trypan blue exclusion method.

(B-C) The percentage of CD4⁺ and CD8⁺ T cells in the lungs and lymph nodes of IL-15^{-/-} and wild-type mice was analyzed by flow cytometry gating on lymphocyte population by forward and side scatter.

(D) The activation status of CD4⁺ and CD8⁺ T cells was assessed by staining lung cells with anti-CD4, anti-CD8, and anti-CD69 antibodies. The results present the percentage of CD69 positive cells within CD4⁺ and CD8⁺ gates.

The data are presented as mean \pm SEM of four mice per group, and the experiments were repeated three times. Statistical analysis was determined by two-sided Student's t-test, where * $p \leq 0.05$, and ** ≤ 0.01 .

6.3.3. Proliferation and apoptosis of CD8⁺ T cells during *M. tuberculosis* infection

IL15^{-/-} or IL15Rα^{-/-} mice are deficient in peripheral CD8⁺, but not in CD4⁺ T cells (254, 255, 276) (and figure 23B and 23C). IL-15 signaling promotes the survival of naïve CD8⁺ T cells by inducing expression of anti-apoptotic proteins, such as Bcl-2 (276, 277). The lack of IL-15 signaling could explain the initial CD8⁺ T cell deficiency in uninfected IL-15^{-/-} mice. Since IL-15 is also important for stimulating proliferation and survival of antigen-specific memory CD8⁺ T cells (CD8⁺ CD44^{hi}) (254, 255, 263), we sought to determine whether proliferation of CD8⁺ T cells was impaired in the absence of IL-15 and whether CD8⁺ T cells were more prone to apoptosis after *M. tuberculosis* infection in IL-15 deficient environment.

Proliferation of CD8⁺ T cells in IL-15^{-/-} and wild-type mice was determined by flow cytometry. Sixteen hours prior to each experiment, mice were injected with BrdU intraperitoneally, and the percentage of BrdU⁺ cells within CD8⁺ gate was determined (Figure 24A). Our data indicate that CD8⁺ T cells did not show any defects in proliferation in response to *M. tuberculosis* infection. A significantly higher percentage of proliferating CD8⁺ T cells in IL-15^{-/-} mice was detected during acute and chronic infection, suggesting that even in the face of persistent exposure to *M. tuberculosis* antigens in IL-15 deficient mice CD8⁺ T cell did not lose their capacity to proliferate (Figure 24A). There were no differences in proliferation of CD8⁺ T cells from the lymph nodes of IL-15^{-/-} and wild-type mice (data not shown).

Since CD8⁺ T cells may be more sensitive to activation induced death in the absence of IL-15, we measured the amount of apoptosis in the CD8⁺ T cell population of IL-15^{-/-} and wild-type mice by staining lung cells with annexin V and 7-AAD. Apoptotic CD8⁺ T cells were defined as being annexin V and 7-AAD positive. Our results indicate that similar percentages of

lung CD8⁺ T cells from IL-15^{-/-} and wild-type mice underwent apoptosis following *M. tuberculosis* infection (Figure 24B). The dynamic T cell responses in the lungs over time, with waxing and waning of proliferation and apoptosis have been previously described (Chapter 2)

Collectively, these findings suggest that the remaining CD8⁺ T cells in IL-15^{-/-} mice were capable of proliferating and were not more susceptible to apoptosis than wild-type CD8⁺ T cells following *M. tuberculosis* infection.

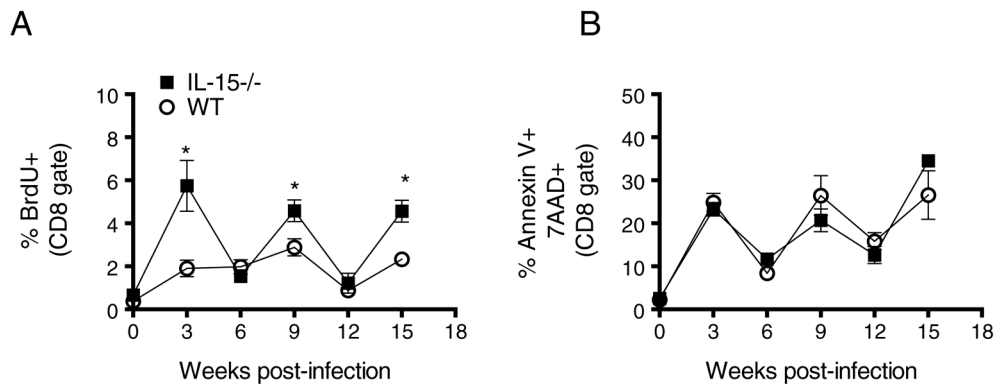


Figure 24 CD8⁺ T cells from IL-15^{-/-} mice are not impaired in proliferation and do not undergo enhanced apoptosis following *M. tuberculosis* infection

(A) To estimate the proliferative capacity of CD8⁺ T cells inside the lungs of IL-15^{-/-} and wild-type mice, BrdU was injected intraperitoneally 16 hours prior to each experiment. Single lung suspensions were stained with anti-CD8 and anti-BrdU antibodies. The results represent mean percentage of proliferating (BrdU⁺) cells within CD8⁺ T cell gate \pm SEM. Statistical significance was determined by two-sided Student's t-test, where * $p \leq 0.05$.

(B) The amount of apoptosis occurring within CD8⁺ T cell population was determined after staining lung cells with anti-CD8 antibody, annexin V and 7-AAD. The results present the percentage of annexin V and 7-AAD positive cells within CD8⁺ T cell gate \pm SEM. No significant differences were observed in the percentage of apoptotic cells within CD8⁺ T cells of IL-15^{-/-} and wild-type mice.

6.3.4. IFN- γ production by CD4⁺ and CD8⁺ T cells in IL-15^{-/-} mice

The number of IFN- γ producing CD4⁺ and CD8⁺ T cells was determined after *ex vivo* stimulation of lung cells with *M. tuberculosis*-infected dendritic cells in the presence of blocking anti-MHC Class I and anti-MHC Class II antibodies, respectively. During acute infection there were no differences in the numbers of IFN- γ producing CD4⁺ and CD8⁺ T cells in the lungs of IL-15^{-/-} and wild-type mice (Figure 25A and 25B). In chronic infection, the number of IFN- γ producing CD4⁺ and CD8⁺ T cells increased in IL-15^{-/-} mice, which in part could be due to increased bacterial burden in the lungs of IL-15^{-/-} mice. Although there were significantly fewer CD8⁺ T cells overall in the IL-15^{-/-} mice the number of IFN- γ producing CD8⁺ T cells was similar between IL-15^{-/-} and wild-type mice. These findings indicate that long-lasting effector CD8⁺ T cells can develop in the absence of IL-15 during *M. tuberculosis* infection.

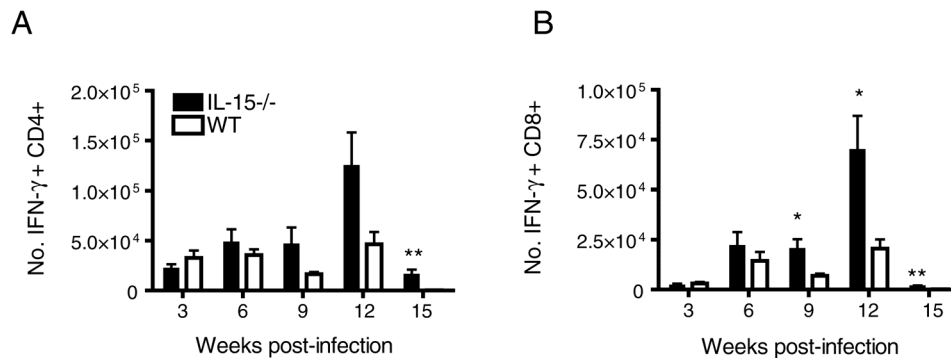


Figure 25 IFN- γ production by CD4⁺ and CD8⁺ T cells isolated from the lungs of IL-15^{-/-} and wild-type mice after *M. tuberculosis* infection

(A-B) The number of IFN- γ producing CD4⁺ and CD8⁺ T cells was determined by ELISPOT assay after *ex vivo* stimulation of lung cells with *M. tuberculosis*-infected dendritic cells incubated with blocking anti-MHC Class I or anti-MHC Class II antibodies, respectively. The background number of spot forming units (SFU) after incubation of lung cells with uninfected dendritic cells was subtracted before calculations were made. The number of IFN- γ producing T cells was determined by multiplying the frequency of IFN- γ producing CD4⁺ or CD8⁺ T cells with total number of lung cells. The results represent the mean number of IFN- γ producing cells \pm SEM of four mice per group. The statistical significance was determined by two-sided Student's t-test, where * $p \leq 0.05$.

6.3.5. Cytotoxic activity of *M. tuberculosis*-specific CD8⁺ T cells in the absence of IL-15

The cytotoxic potential of IL-15^{-/-} and wild-type CD8⁺ T cells was determined by limiting dilution analysis, using T cells harvested from the lungs, and tested in an *M. tuberculosis* specific assay (240, 241) (Chapter 2). The data are summarized as mean number of CTL precursors per lung for each group of mice (Figure 26). Our results demonstrate that the cytotoxic CD8⁺ T cells develop and function normally in IL-15^{-/-} deficient environment during acute infection. Both groups of mice followed the same kinetics of cytotoxic activity, which in murine model of tuberculosis is characterized by high cytotoxic activity during acute infection, and loss of cytolytic CD8⁺ T cells during chronic infection (Chapter 2). Although there were significantly more CTLp in the lungs of IL-15^{-/-} mice 9 weeks post-infection, the cytotoxicity was negligible in both groups of mice by 12 weeks post-infection (Figure 26).

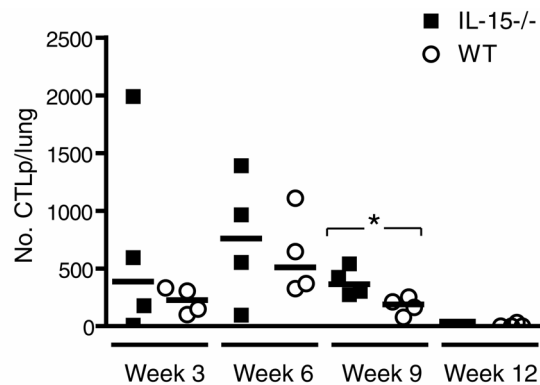


Figure 26 Cytotoxic CD8⁺ T cells are functional in the absence of IL-15

The frequency of CTLp in the lungs of IL-15^{-/-} and wild-type mice was determined by limiting dilution analysis (LDA). Serial dilutions of lung cells (40,000 cells/well → 1250 cells/well; 24 replicates/cell dilution) were stimulated with *M. tuberculosis*-infected dendritic cells for seven days, followed by another round of stimulation with *M. tuberculosis*-infected macrophages. After 14 days of expansion, the cytotoxicity of each individual well was determined by chromium release assay using *M. tuberculosis* infected macrophages as targets. The actual frequency of CTLp was determined by χ^2 minimization analysis. The experiments were repeated twice with similar results.

6.3.6. IL15^{-/-} mice successfully control secondary infection with *M. tuberculosis*

Resistant C57BL/6 wild-type mice control *M. tuberculosis* infection, but they are unable to completely eliminate the bacteria even in the face of potent immune response. To study memory responses mycobacteria were cleared prior to challenge by treating IL-15^{-/-} and wild-type mice with a combination of pyrazinamide (15 g/l in drinking water) and isoniazid (0.1 g/l) beginning four weeks post-infection for two months. Prolonged treatment with this combination of antibiotics was previously shown to have sterilizing activity in wild-type mice (47). Although wild-type mice cleared the infection at the end of a 2-month antibiotic treatment, *M. tuberculosis* still persisted in IL-15^{-/-} mice. For this reason, the antibiotic regimen was changed to isoniazid (0.1 g/l) and rifampicin (0.15 g/l) for additional 4 months until plating of whole lung homogenates revealed no viable mycobacteria. Mice were challenged with a low dose of *M. tuberculosis* via aerosol route, and the ability of immune IL-15^{-/-} and wild-type mice to control secondary *M. tuberculosis* infection was evaluated. Both immune IL-15^{-/-} and wild-type mice were equally efficient at controlling bacterial burden after *M. tuberculosis* challenge (Figure 27).

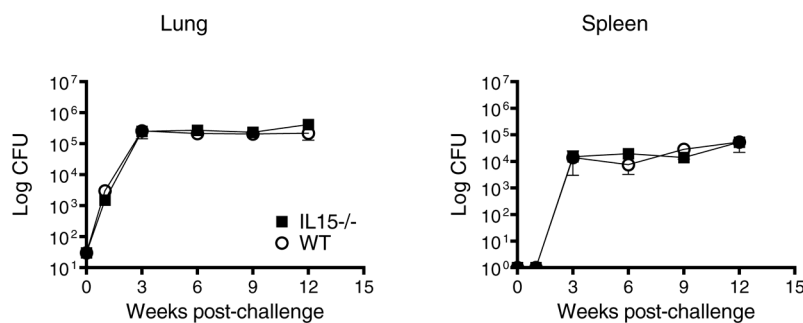


Figure 27 IL-15^{-/-} mice successfully controlled secondary *M. tuberculosis* infection

IL-15^{-/-} and wild-type mice were infected with a low dose *M. tuberculosis* infection (primary infection). Prolonged antibiotic treatment, which commenced at 4 weeks post-infection and lasted for 6 months, ensured complete elimination of mycobacteria and establishment of a memory pool of CD4⁺ and CD8⁺ T cells. After resting period, IL-15^{-/-} and wild-type mice were challenged with a low dose of *M. tuberculosis* via aerosol route (secondary infection). The numbers represent mean \pm SEM of four mice per group.

6.3.7. Memory CD4⁺ and CD8⁺ T cell responses in IL-15^{-/-} mice

Previous reports have demonstrated that IL-15^{-/-} or IL-15R α ^{-/-} mice have reduced numbers of memory CD8⁺ T cells (254, 255), mainly due to decreased proliferation and decreased homing of IL-15R α ^{-/-} lymphocytes to peripheral lymph nodes (255). We investigated the importance of IL-15 in the development of *M. tuberculosis*-specific T cell memory following challenge with a low dose of *M. tuberculosis*. Infiltration of immune cells into the lungs of IL-15^{-/-} mice was delayed at 3 weeks post-challenge, but by 6 weeks post-challenge infiltration of cells into the lungs of IL-15^{-/-} and wild-type mice was similar (Figure 28A). No apparent differences were observed in lymph node cell numbers between two groups of mice throughout the secondary infection (Figure 28A). Although there were similar percentages of CD4⁺ T cells in the lymph nodes, infiltration of CD4⁺ T cells into the lungs of IL-15^{-/-} mice initially lagged behind wild-type mice (Figure 28B). The absence of IL-15 resulted in significantly lower percentage of activated CD69⁺ CD4⁺ T cells after secondary challenge (Figure 28D). The response of IL15^{-/-} CD8⁺ T cells was very similar during primary and secondary *M. tuberculosis* infection. A 2-fold difference in percent CD8⁺ T cells in the lungs and lymph nodes of IL-15^{-/-} mice was also obvious during secondary infection (Figure 28C). Although fewer CD8⁺ T cells were present in the lungs of IL-15^{-/-} mice, equal percentages of activated CD8⁺ T cells were detected in the lungs of both groups of mice after secondary infection with *M. tuberculosis* (Figure 28D).

Differences seen in the number of immune cells within the lungs of immune IL-15^{-/-} mice could be a result of a smaller burst size of effector CD8⁺ T cell responses in IL-15^{-/-} mice during primary infection. Nevertheless, similar percentages of activated CD8⁺ T cells were observed in the lungs of IL-15^{-/-} and wild-type mice after secondary *M. tuberculosis* infection.

Interestingly, infiltration of CD4⁺ T cells was delayed initially in the absence of IL-15, and fewer IL-15^{-/-} CD4⁺ T cells expressed early activation marker CD69 after secondary *M. tuberculosis* infection.

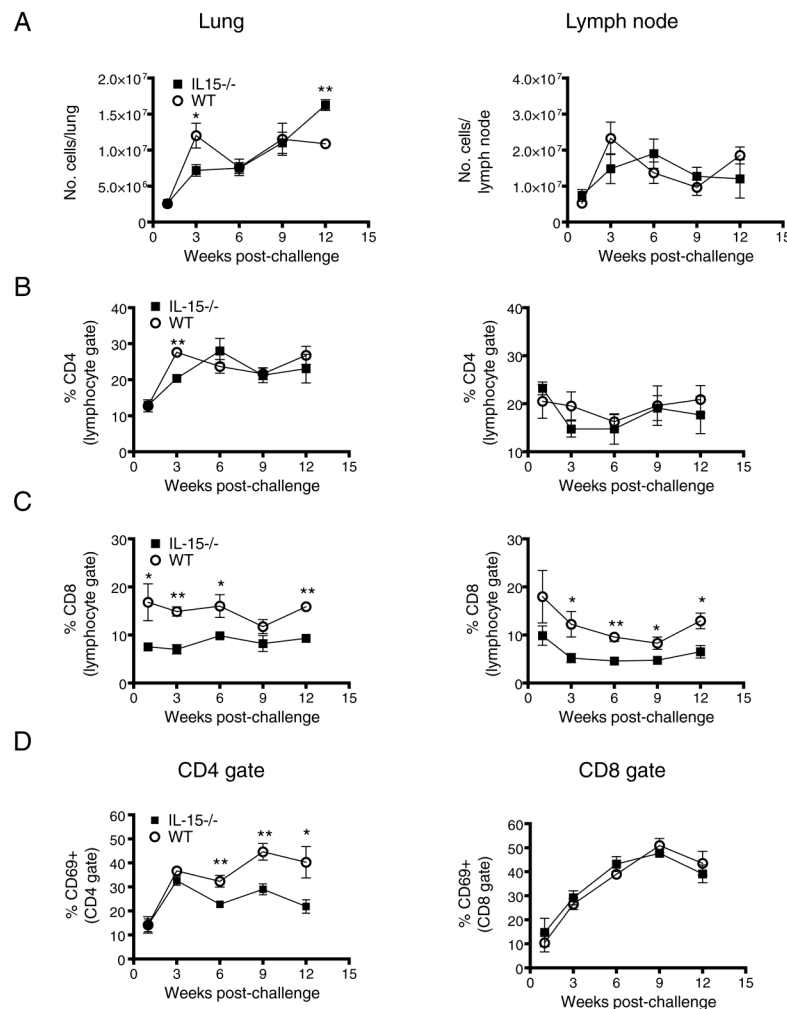


Figure 28 Lung lymphocyte analysis after secondary infection with *M. tuberculosis*

(A) Overall numbers of viable cells in the lungs of IL-15^{-/-} and wild-type mice following *M. tuberculosis* challenge were determined by trypan blue exclusion method.

(B – C) The percentage of CD4⁺ and CD8⁺ T cells in the lungs and lymph nodes of IL-15^{-/-} and wild-type mice was determined by flow cytometry.

(D) The percentage of activated CD4⁺ and CD8⁺ T cells in the lungs of IL-15^{-/-} and wild-type mice was evaluated by staining for CD69 expression using flow cytometry. Data represent mean percentage of CD69⁺ T cells within CD4⁺ and CD8⁺ gates \pm SEM.

6.3.8. Proliferation and cytokine production by memory CD4⁺ and CD8⁺ T cell responses is not impaired in IL-15^{-/-} mice

In contrast to previous findings where numbers of virus specific memory CD8⁺ T cells slowly declined in IL-15 deficient mice (263, 264), the most dramatic difference in memory T cell responses of IL-15^{-/-} and wild-type mice was observed early after secondary infection with *M. tuberculosis*. Immune IL-15^{-/-} mice had significantly fewer CD4⁺ and CD8⁺ T cells until six weeks post-challenge (Figure 29A). This difference in total numbers of CD4⁺ and CD8⁺ T cells during early recall response was not due to impaired proliferation as similar percentages of proliferating CD4⁺ and CD8⁺ T cells were detected in the lungs of immune IL-15^{-/-} and wild-type mice (Figure 29B). These differences are most likely due to delayed infiltration of memory CD4⁺ and CD8⁺ T cells into the infected lungs following secondary *M. tuberculosis* infection.

We assessed the ability of memory CD4⁺ and CD8⁺ T cells from IL-15^{-/-} and wild-type mice to produce IFN- γ in response to *M. tuberculosis*-infected dendritic cells. Equal numbers of IFN- γ producing CD4⁺ and CD8⁺ memory T cells were present in the lungs of immune IL-15^{-/-} and wild-type mice (Figure 29C) suggesting that the IFN- γ production by memory T cells is not dependent on IL-15.

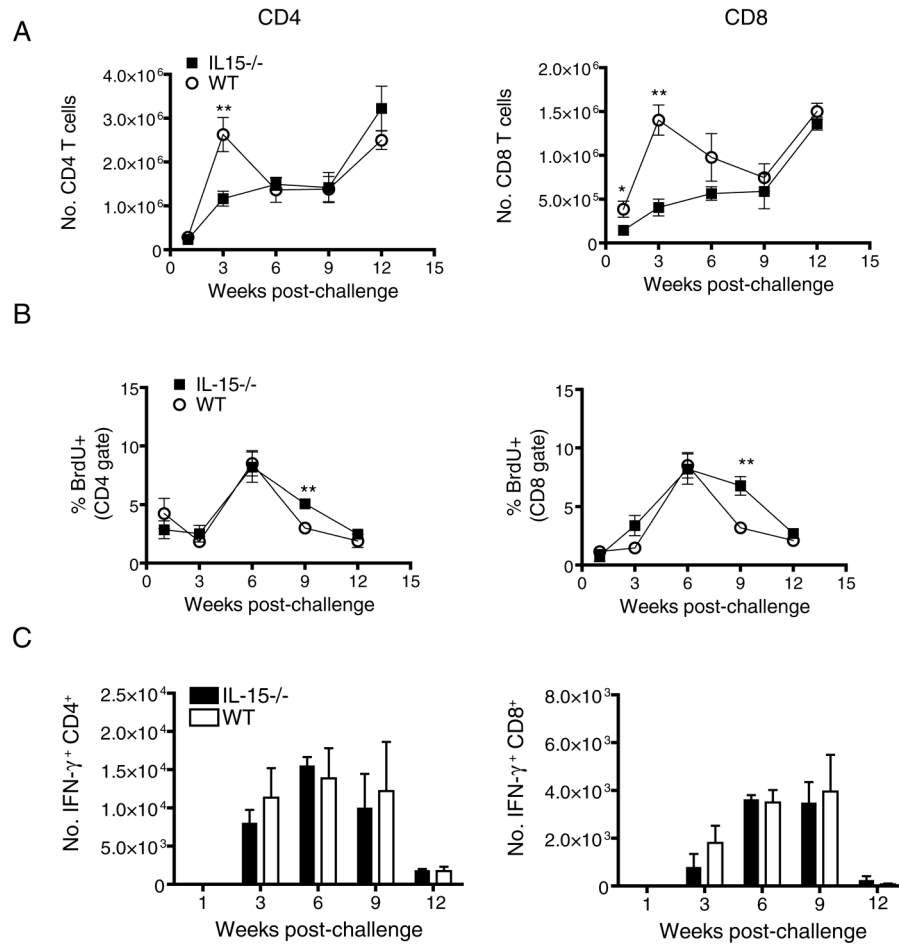


Figure 29 Proliferation and cytokine production by CD4⁺ and CD8⁺ T cells in immune IL-15^{-/-} and wild-type mice

(A) The absolute numbers of CD4⁺ and CD8⁺ T cells were determined after staining lung cells with anti-CD4 and anti-CD8 antibodies. The numbers were calculated by multiplying total number of lung cells with % CD4⁺ or % CD8⁺ within live cell gate. The results are presented as mean ± SEM of four mice per group. The most dramatic difference in immune IL-15^{-/-} and wild-type mice was seen early after secondary infection as infiltration of memory CD4⁺ and CD8⁺ T cells was delayed into infected lungs in the absence of IL-15. Similar numbers of CD4⁺ and CD8⁺ T cells were detected between IL-15^{-/-} and wild-type mice at 3 months post-challenge.

(B) The percentage of proliferating (BrdU⁺) cells within CD4⁺ and CD8⁺ gates was determined as described in Materials and Methods and figure legend 3. The graphs represent mean ± SEM of four mice per group. Statistical differences were calculated using two-sided Student's t-test, where * $p \leq 0.05$, and ** ≤ 0.01 . CD4⁺ and CD8⁺ T cells from immune IL-15^{-/-} and wild-type mice showed similar proliferation kinetics.

(C) The numbers of IFN-γ producing CD4⁺ and CD8⁺ T cells in the lungs of immune IL-15^{-/-} and wild-type mice was determined by ELISPOT using *M. tuberculosis*-infected dendritic cells as antigen specific stimulators. Incubation of *M. tuberculosis*-infected dendritic cells with blocking MHC Class I or MHC Class II antibodies delineated the number of IFN-γ producing CD4⁺ or CD8⁺ T cells, respectively. The inhibitory effect of the blocking MHC Class I and MHC Class II antibodies was ≥ 95% at 10 μg/ml concentration. The calculations were made as described in the figure legend 25. The mean numbers of IFN-γ producing CD4⁺ and CD8⁺ T cells from four IL-15^{-/-} and wild-type mice at each time point are shown. Error bars represent SEM.

6.4. Discussion

The main goal of this study was to investigate whether IL-15 is required for the generation and maintenance of effector and memory T cell responses following *M. tuberculosis* infection. The main findings of this study indicate that IL-15^{-/-} mice were not substantially impaired in their ability to control primary and secondary *M. tuberculosis* infection. Similar numbers of mycobacteria were detected in the lungs, lymph nodes and spleen of IL-15^{-/-} and wild-type mice. There was a tendency for slightly higher bacterial burden in the lungs of IL-15^{-/-} mice during chronic *M. tuberculosis* infection. CD4⁺ and CD8⁺ T cell effector functions were not affected by IL-15 deficiency after primary and secondary *M. tuberculosis* infection. In the view of recent studies, the most relevant finding is that CD8⁺ T cells were not more prone to apoptosis following *M. tuberculosis* infection, and there was no sign of reduced proliferation of effector and memory CD8⁺ T cells in IL-15 deficient mice. The most dramatic difference in immune IL-15^{-/-} and wild-type mice was characterized by delayed infiltration of immune cells into infected lungs early after secondary challenge.

Initial studies in which the potential of IL-15 as an immunotherapeutic agent against *M. tuberculosis* has been investigated suggest that overexpression of IL-15 can augment T cell mediated immune responses against *M. tuberculosis* (273-275). Infection of IL-15 transgenic mice with *M. bovis* BCG conferred better protection against challenge with virulent *M. tuberculosis* H37Rv than *M. bovis* BCG alone (274). This enhanced efficacy of BCG by IL-15 could be attributed in part to enhanced CD8⁺ T cell responses (274). However, exogenous IL-15 administration did not increase the survival or improved the effector function of CD8⁺ T cells in susceptible CD4^{-/-} mice (C57BL/6 background) after low dose infection with aerosolized *M. tuberculosis* (see Appendix A). Furthermore, IL-15 treatment did not change the course of infection in wild-type mice (see Appendix A).

In this study we sought to determine whether IL-15 was required for the generation of primary and recall T cell responses against *M. tuberculosis*. The baseline number of CD8⁺ T cells in the lungs and lymph nodes in uninfected IL-15^{-/-} mice was at least 2-fold lower when compared to uninfected wild-type mice. Initial studies with IL-15^{-/-} and IL-15R α ^{-/-} mice revealed that in the absence of IL-15 signaling there was ~ 30 – 50% reduction in peripheral CD8⁺ T cells (254, 255). Additional studies revealed that IL-15 signaling is important for the survival of peripheral naïve CD8⁺ T cells, which is most likely mediated by increased expression of anti-apoptotic proteins, such as Bcl-2 (276, 277). Since the initial CD8⁺ T cell deficiency in IL-15^{-/-} mice could be explained by reduced survival of naïve T cells associated with IL-15-deficient mice, there was still a possibility that a reduction in the overall CD8⁺ T cell effector population after primary infection could be due to reduced proliferation or enhanced apoptosis of CD8⁺ T cells in the absence of IL-15. We tested these hypotheses, and our data indicate that significantly higher percentage of effector CD8⁺ T cells proliferated in IL-15^{-/-} mice during acute and chronic *M. tuberculosis* infection. It is important to emphasize that the proliferative ability of CD8⁺ T cells in the face of persistent stimulation with *M. tuberculosis* antigens is not undermined in the absence of IL-15. Since CD8⁺ T cells may be more prone to undergo apoptosis after antigenic stimulation in the absence of IL-15, we determined the percentage of apoptotic CD8⁺ T cells during acute and chronic *M. tuberculosis* infection. Staining of lung cells with annexin V and 7-AAD revealed a similar percentage of apoptotic CD8⁺ T cells between IL-15^{-/-} and wild-type mice. Therefore, the low magnitude CD8⁺ T cells responses during primary *M. tuberculosis* infection of IL-15^{-/-} mice is not a result of reduced proliferation or enhanced apoptosis. Furthermore, the cytokine production and cytotoxic activity of CD8⁺ T cells were

normal in IL-15^{-/-} mice, suggesting that IL-15 is not required for the effector functions of *M. tuberculosis*-specific CD8⁺ T cells.

In accordance with previous studies (254, 255), CD4⁺ T cell responses were not affected by the absence of IL-15 signaling. Similar percentages of proliferating and apoptotic CD4⁺ T cells were detected in the lungs and lymph nodes of IL-15^{-/-} and wild-type mice (data not shown). The effector function of CD4⁺ T cells was normal in IL-15^{-/-} mice as evidenced by a high percentage of activated CD4⁺ T cells and potent IFN- γ CD4⁺ T cell responses.

Interestingly, the number of IFN- γ producing CD4⁺ and CD8⁺ T cells increased in the lungs of IL-15^{-/-} mice during chronic infection compared to wild-type mice. This boosted IFN- γ response could be ascribed to increased bacterial burden in the lungs of IL-15^{-/-} mice. Notably the number of IFN- γ producing CD4⁺ and CD8⁺ T cells did not decline in the absence of IL-15.

There are no phenotypic markers that reproducibly represent the functional characteristics of memory CD4⁺ and CD8⁺ T cells in murine model of tuberculosis. Combination of CD44, Ly6C, CD62L markers failed to clearly delineate memory cells from effector cells after *M. tuberculosis* infection of naïve or memory mice (Chapter 4). The only distinguishing feature of memory response in murine model of tuberculosis is a higher percentage of activated CD69⁺ T cells in the lungs of immune mice when compared with naïve mice within the first 3 weeks post-challenge (Chapter 4). Our data indicate that protective immune responses were generated in the absence of IL-15 as evidenced by strict control of bacterial loads in the lungs and spleens of challenged IL-15^{-/-} and wild-type mice.

Several groups reported that in the absence of IL-15 or IL-15R α signaling the homeostatic proliferation of memory CD8⁺ T cells was significantly diminished leading to a slow decline in virus-specific memory CD8⁺ T cells (263, 264). There was no indication of

reduced proliferation or a decline in memory CD4⁺ and CD8⁺ T cell responses in IL-15^{-/-} mice in our studies. The most dramatic difference between immune IL-15^{-/-} and wild-type mice was observed early after secondary challenge with *M. tuberculosis*. There was a significant delay in the infiltration of CD4⁺ and CD8⁺ T cells into the lungs of IL-15^{-/-} immune mice at three weeks post-challenge. Alternatively, initial differences could also be attributed to a smaller burst size of effector CD8⁺ T cell responses during primary infection of IL-15^{-/-} mice. By six weeks post-challenge the kinetics and magnitude of memory T cell responses were similar between IL-15^{-/-} and wild-type mice. As *M. tuberculosis* is not cleared by a secondary immune response, and reinfection is established, it is important to note that any effect of IL-15^{-/-} deficiency on maintenance of memory T cell responses could be masked by the influx of *de novo* primed effector T cells at 6 weeks post-infection.

Although development of primary T cell responses occurred normally in IL15^{-/-} mice, these mice had difficulty clearing *M. tuberculosis* infection when treated with antibiotics. We observed previously that CD4^{-/-} mice were unable to eliminate *M. tuberculosis* during antibiotic treatment (Chapter 4). Since CD4⁺ T cells play a pivotal role in controlling acute and persistent *M. tuberculosis* infection, the efficacy of antibiotics was undermined in the absence of this important T cell subset. These findings suggest antibiotics treatment must be prolonged in the cases of immunodeficiency such as absence of CD4⁺ T cells. Since IL-15^{-/-} mice had a similar difficulty in eliminating mycobacteria during antibiotic treatment suggested that this cytokine may have other unidentified roles in modulating the immune system that may be overlooked using standard immunological techniques. Flow cytometric analysis revealed similar numbers of dendritic cells, neutrophils and macrophages in the lungs of IL-15^{-/-} and wild-type mice and cytokine profile analysis revealed no significant deficiencies in IL-2, IL-7, IL-12 and IL-10

mRNA expression (data not shown). Since no major differences in the functionality of T cells, composition of innate immune cells, and cytokine profile were detected in IL15^{-/-} mice, it still remains unresolved why IL-15^{-/-} mice were unable to clear *M. tuberculosis* infection in the presence of a standard course of antibiotics.

Collectively, our results indicate that IL-15 is not essential for the generation and maintenance of effector CD4⁺ and CD8⁺ T cell responses. The magnitude of the recall response in immune IL-15^{-/-} mice was significantly smaller than in immune wild-type mice, which could be due to delayed infiltration of immune cells or a consequence of a smaller burst size of effector CD8⁺ T cell responses during primary infection of IL-15^{-/-} mice. Development of tetramer staining reagents for *M. tuberculosis* antigens and better phenotypic definition of *M. tuberculosis*-specific memory T cells will enable a detailed investigation of development and maintenance of memory T cell responses following secondary *M. tuberculosis* infection.

7. CHAPTER 4 CD4⁺ T CELLS ARE NECESSARY FOR SUSTAINED CD8⁺ T CELL MEMORY RESPONSES FOLLOWING CHALLENGE WITH *MYCOBACTERIUM TUBERCULOSIS*

7.1. Introduction

The contribution of CD8⁺ T cells to control of *M. tuberculosis* infection was first established through adoptive transfers and by *in vivo* CD8⁺ T cell depletion experiments (139-141). Subsequently, it was demonstrated that mice which lack functional classically and non-classically restricted CD8⁺ T cells were more susceptible to *M. tuberculosis* infection than wild-type mice as evidenced by increased bacterial burden and reduced survival (142, 143, 278). In wild-type mice *M. tuberculosis*-specific CD8⁺ T cells emerged early in infection, produced inflammatory cytokines, such as IFN- γ and TNF- α , expressed perforin and lysed *M. tuberculosis*-infected macrophages in a β_2m – and MHC Class I-dependent manner (130, 146). Although priming, migration and IFN- γ production by CD8⁺ T cells were normal in CD4⁺ T cell-deficient mice during acute *M. tuberculosis* infection, cytotoxic CD8⁺ T cell responses were impaired in the absence of CD4⁺ T cell help (138).

The role of CD4⁺ T cells in the induction and maintenance of effector CD8⁺ T cell responses and in generation of CD8⁺ T cell memory has been extensively investigated. Studies using different stimuli (inflammatory versus non-inflammatory), or different systems (infection versus cross-priming) led to the definition of helper-dependent and helper-independent CD8⁺ T cell responses. In viral systems, CD4⁺ T cell help was not required for the induction of primary CD8⁺ T cell responses with viruses that are cleared efficiently (151, 279). However, in the case of chronic viral infections, CD8⁺ T cell responses atrophied in the absence of CD4⁺ T cells, and failure to sustain functional CD8⁺ T cell responses resulted in impaired viral clearance (229, 235,

280-282). Inflammatory nature of an antigen (222, 283-288), route of immunization (289), CTLp frequency (285, 290) and epitope affinity for MHC Class I molecules (291) have all been reported to influence the helper dependence of cytotoxic CD8⁺ T cells.

CD4⁺ T cells could provide help to CD8⁺ T cells either through “conditioning” of antigen presenting cells (APCs) via CD40-CD40L interaction, or by influencing the availability of cytokines, such as IL-2, IL-12 and IL-15, which are essential for the normal functioning of CD8⁺ T cells. Although several studies reported the importance of soluble factors for normal CD8⁺ T cell differentiation (292-297), it appears that signaling through CD40 molecule on APCs is possibly the most important helper-dependent mechanism for CTL generation. In support of this hypothesis, extensive studies demonstrated that CD40 and CD40L deficiencies resulted in impaired CD8⁺ T cells responses, and anti-CD40 stimulating antibodies could substitute for CD4⁺ T cell help (210, 221, 222).

Although CD4⁺ T cells may or may not be required for CD8⁺ T cell activation, division and differentiation into effector cells, several studies reported that CD4⁺ T cells are absolutely essential for providing instructive signals to CD8⁺ T cells to differentiate into effective memory cells (298-301). The unifying theme from these studies is that quality of memory CD8⁺ T cell responses is dependent on CD4⁺ T cell help. The most striking difference between “helped” and “unhelped” memory CD8⁺ T cells was in their ability to proliferate (299-301). Memory CD8⁺ T cells generated in the absence of CD4⁺ T cells failed to expand in response to secondary challenge, and the amount of IFN- γ and IL-2 production was significantly diminished when compared to memory CD8⁺ T cells that developed with CD4⁺ T cell help (299-301). Although CD4⁺ T cell help was most important during priming of CD8⁺ T cells responses (299-301), CD4⁺ T cells may also influence the properties of memory CD8⁺ T cells at later stages in infection.

Previously, we showed that CD8⁺ T cells participate in the recall responses to *M. tuberculosis* challenge. *M. tuberculosis*-specific memory CD8⁺ T cells were rapidly mobilized into the lungs of immune mice one week post-challenge. In contrast to naïve CD8⁺ T cells, memory CD8⁺ T cells exhibited swift up-regulation of CD69 (early activation marker), and enhanced IFN- γ production (302). These findings underscore the importance of incorporating stimulation of CD8⁺ T cell responses into rational vaccine design against tuberculosis. Therefore, it is necessary to define the requirements for generation and maintenance of effective memory CD8⁺ T cell responses that will lead to better protection against this pathogen. In this study, we investigated the role of CD4⁺ T cells in the maintenance of memory CD8⁺ T cells. Our findings indicate that although expansion and proliferation of memory CD8⁺ T cells was normal in the absence of CD4⁺ T cells, the IFN- γ producing memory CD8⁺ T cells were unable to persist after 6 weeks post-challenge. The results from this study emphasize the importance of CD4⁺ T cells in maintaining effective memory IFN- γ producing CD8⁺ T cells.

7.2. Materials and Methods

7.2.1. Mice and infections

C57BL/6 wild-type mice were purchased from Charles River Laboratories (Wilmington, MA). CD4^{-/-} mice were bred at the University of Pittsburgh Biotechnology Center. All mice were kept under specific pathogen-free conditions in a Biosafety Level 3 facility. Animal protocols used in this study were approved by the University Institutional Animal Care and Use Committee. For the primary infection and secondary challenge mice were infected with a low dose of aerosolized *M. tuberculosis* (Erdman strain, Trudeau Institute, Saranac Lake, NY), achieved with 5×10^5 /ml in the nebulizer of a nose-exposure only aerosolizer unit (Intox Inc., Moriarty, NM). The dose received was estimated by plating whole lung homogenates of two mice from each experiment 24 hours following aerosol infection.

7.2.2. *In vivo* CD4 depletion and establishment of *M. tuberculosis*-specific memory models

In general, mice are infected with a low dose of *M. tuberculosis* (~30 CFU/lung) via aerosol route. Infection proceeds for four weeks to allow for the induction of primary CD8⁺ T cell responses. From 4 weeks post-infection, mice are treated with isoniazid (0.1 g/liter) and pyrazinamide (15 g/liter) in drinking water two times a week for 8 weeks to clear the infection. At the end of antibiotic treatment two mice are sacrificed and several organ homogenates (lung and spleen) are plated on 7H10 plates (Difco) to confirm the absence of viable mycobacteria. Mice are rested for 16 weeks before secondary challenge with a low dose of *M. tuberculosis* via aerosol route. The quality of CD8⁺ memory T cell responses were investigated at this stage in the memory model.

In the first memory model (Figure 30A and 30B), mice were injected with 500 µg/mouse of GK1.5 (anti-CD4) antibody to deplete CD4⁺ T cells one week before infection, and anti-CD4 antibody treatment was continued twice a week for four weeks (Figure 30A) or 12 weeks (Figure 30B). Antibiotic treatment began at four weeks post-infection and lasted for 8 weeks. The anti-CD4 depletion regimen resulted in >96% depletion of CD4⁺ T cells. The remaining <4% CD4⁺ T cells were CD4^{low} and failed to produce IFN-γ following non-specific stimulation with anti-CD3/CD28 antibodies (data not shown).

In the second memory model (Figure 30C) wild-type mice were infected with a low dose of *M. tuberculosis* for four weeks. Antibiotic treatment was initiated at 4 week post-infection for the following 8 weeks. Mice were rested for 16 weeks, and CD4⁺ T cells were depleted one week prior to the secondary challenge with 500 µg/mouse of GK1.5 antibody. The CD4 depletion regimen was continued twice weekly for the duration of study (9 weeks post-challenge). Naive CD4^{-/-} and wild-type mice were infected and included as additional controls.

7.2.3. CFU determination

Lung homogenates were serially diluted in PBS/0.05% Tween-80 and plated on 7H10 agar plates (Difco). Plates were incubated at 37°C, 5% CO₂ for 21 days prior to counting colonies.

7.2.4. Bone marrow derived macrophages and dendritic cell cultures

In *ex vivo* stimulation assays, such as ELISPOT and limiting dilution analysis, bone marrow derived dendritic cells were cultured in the presence of GM-CSF supernatant at 1:200 dilution (a generous gift from Dr. Binfeng Lu, University of Pittsburgh) and 20 ng/ml of IL-4

(PeproTech Inc, Rocky Hill, NJ) and macrophages were cultured in the presence of L cell supernatant as a source of CSF-1 using standard procedures described previously (146).

7.2.5. Flow cytometry for cell surface molecules

Lung single cell suspensions were stained as described previously (146). Cells were stained with anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7), anti-CD69 (clone H1.2F3), anti-Ly6C (clone AL-21), anti-CD44 (clone IM7), and anti-CD62L (clone MEL-14) fluorescently conjugated antibodies. All antibodies were purchased from BD Pharmingen (San Diego, CA) and used at 0.2 µg/ml concentration. Cells were collected on a FACSCaliber (Beckon Dickinson, San Jose, CA) and analyzed by CellQuest (Becton Dickinson) or FlowJo (Tree Star Inc, San Carlos, CA) software.

7.2.6. Proliferation of T cells in the lungs of infected mice

Sixteen hours prior to each experimental time point, mice were injected intraperitoneally with saline containing 1 mg of 5-bromo-2'-deoxyuridine [BrdU] (Sigma-Aldrich, St. Louis, MO). Lung cells were stained for cell surface markers CD4 and CD8 at room temperature for 20 minutes prior to a fixation step with 4% paraformaldehyde [PFA] (200 µl/tube) on ice for 20 minutes. Cells were washed with tissue culture phosphate buffered saline [PBS] at 470 g, and cell pellets were suspended in ice-cold 0.15 M NaCl (100 µl/tube), followed immediately by drop-wise fixation with ice-cold 95% ethanol (200 µl/tube) on ice for 30 minutes. Following a PBS wash, cells were permeabilized and fixed with 200 µl/tube of 0.4% saponin and 2% PFA for 1 hour at room temperature. Cells were washed with PBS, and suspended in 200 µl/tube of 0.15 M NaCl, 4.2 mM MgCl₂ (pH 5) containing 250 U/ml of DNase I (Roche, Indianapolis, IN) for

30 minutes in 37°C water bath. Cells were washed with PBS, and incubated with anti-BrdU antibody or the respective isotype control (BD Pharmingen, FITC-conjugated antibody set) diluted at 1:3 in 0.5% Tween-20 and 0.5% BSA (50 µl/tube). Following 30 minute incubation at room temperature, cells were washed with PBS, and fixed with 4% PFA prior to acquisition on the flow cytometer.

7.2.7. IFN- γ production

Cytokine production by CD8⁺ T cells isolated from the lungs of *M. tuberculosis* infected mice was evaluated by ELISPOT as described previously (228). Briefly, lung and lymph node cells were plated in anti-IFN- γ antibody (BD Pharmingen, clone R4-6A2) coated plates (MAIPS4510, Millipore Corp, Bedford, MA) at 80,000 cells/well and 150,000 cell/well, respectively. Cells were incubated in duplicate wells with media, ConA (10 µg/ml; Sigma-Aldrich), uninfected and *M. tuberculosis*-infected dendritic cells (MOI 3; overnight) to estimate the number of total number of IFN- γ producing T cells, and *M. tuberculosis*-infected dendritic cells incubated with the blocking anti-MHC Class I (BD Pharmingen, clone 8F12) or anti-MHC Class II (BD Pharmingen, clone M5/114.15.2) antibodies at 10 µg/ml to estimate the number of IFN- γ producing CD4⁺ and CD8⁺ T cells, respectively. All dendritic cells were added to lung and lymph nodes cells at 1:2 ratio and the cultures were supplemented with IL-2 (PeproTech) at final concentration of 20 U/ml.

Following 40 hour incubation, the IFN- γ producing T cells were visualized after stepwise incubation of plates with biotinylated anti-IFN- γ antibody (BD Pharmingen, clone XMG 1.2) , streptavidin-conjugated enzyme (PK-6100, Vector Laboratories) and AEC substrate (SK-4200, Vector Laboratories). The spot forming units (SFU) were enumerated using ELISpot reader

(Cellular Technology Ltd, Cleveland, OH). The number of IFN- γ producing CD8⁺ T cells was determined using the following formula: (total number of cells/lung) x (frequency of IFN- γ producing cells after stimulation with *M. tuberculosis* infected dendritic cells pre-incubated with anti-MHC Class II antibody). Background number of IFN- γ producing cells following stimulation with uninfected dendritic cells (< 10 SFU) was subtracted before calculations were made.

7.2.8. Limiting dilution analysis

The cytotoxic potential of CD8⁺ T cells was estimated using limiting dilution analysis (LDA) (240, 241). Effector cells were derived from the lungs and lung draining lymph nodes of *M. tuberculosis*-infected mice at designated time points. Freshly isolated cells were plated in 2-fold serial dilutions starting from 40,000 cells/well to 1250 cells/well in V-bottom 96-well plates (24 replicates/input number) supplemented with IL-2 at 20 U/ml. Lung and lymph node cells were incubated with *M. tuberculosis*-infected dendritic cells (500 DC/well) for 7 days. Following incubation, 100 μ l of media was removed from each well, and lung cells were cultured for another round of stimulation with *M. tuberculosis*-infected macrophages (1000 M Φ /well) and IL-2 (20 U/ml) to allow for expansion of CTL precursors. Flow cytometry analysis revealed that 75-95% of cells were CD8⁺ T cells after 2 weeks of stimulation.

Cytotoxicity was determined in each well by a standard ⁵¹Chromium release assay with *M. tuberculosis*-infected macrophages as targets. *M. tuberculosis*-infected macrophages were labeled with ⁵¹Cr (100 μ l of ⁵¹Cr per 3x10⁶ macrophages) for 1 hour at 37°C, and added to lung and lymph node T cell cultures at 4000 cells/well. Following 4 hour incubation, 100 μ l of supernatant was collected (Skatron SCS System; Skatron, Sterling, VA) and radioactivity was

quantified using a gamma counter. Positive wells were defined as being greater than mean + 3 SD of spontaneous target cell release. Frequency of CTLp was determined using zero-order Poisson equation ($\ln Y = -Fx + \ln A$; where x = the number of effector cells/well; Y = % negative wells; A = the y-axis intercept; F = CTLp frequency defined by the negative slope of the line). All calculations were performed using a software program fitted to the equation by χ^2 minimization analysis (a generous gift from Dr. Carolyn A. Keever-Taylor, Medical College of Wisconsin).

7.2.9. Statistics

Statistically significant differences in the numbers of effector T cells between the CD4 depleted and wild-type mice were determined using unpaired, two-sided, Student t-test. The p-value of < 0.05 was defined as being significant. Designations in the figures: * $p \leq 0.05$; ** $p \leq 0.01$.

7.3. Results

7.3.1. Effects of CD4⁺ T cell depletion during primary infection on the quality of memory CD8⁺ T cell responses

In the first memory model (Figure 30A) we addressed the importance of CD4⁺ T cells during priming of CD8⁺ T cell responses for the subsequent generation of CD8⁺ T cell memory. CD4⁺ T cells were depleted one week prior to *M. tuberculosis* infection and during the first four weeks of primary infection to allow for the priming of effector CD8⁺ T cell responses. Antibiotic treatment with isoniazid (0.1 g/l in drinking water) and pyrazinamide (15 g/l) commenced at four weeks post-infection and continued for eight weeks. In accordance with previously published studies such antibiotic treatment of C57BL/6 wild-type mice infected with an aerosolized low dose of *M. tuberculosis* resulted in the complete elimination of the bacilli as assessed by plating whole lung homogenates (47, 302, 303). In addition, immunosuppression of such antibiotic treated mice failed to result in reactivation suggesting that complete sterilization of the lungs was achieved (47). Mice were rested for 16 weeks prior to secondary challenge with *M. tuberculosis*. The absence of mycobacteria and a long resting period ensured that a stable pool of memory CD8⁺ T cells was established (302). Twenty-eight weeks after initial infection, mice were challenged with a low dose of *M. tuberculosis* via aerosol route, and memory CD8⁺ T cell responses were evaluated.

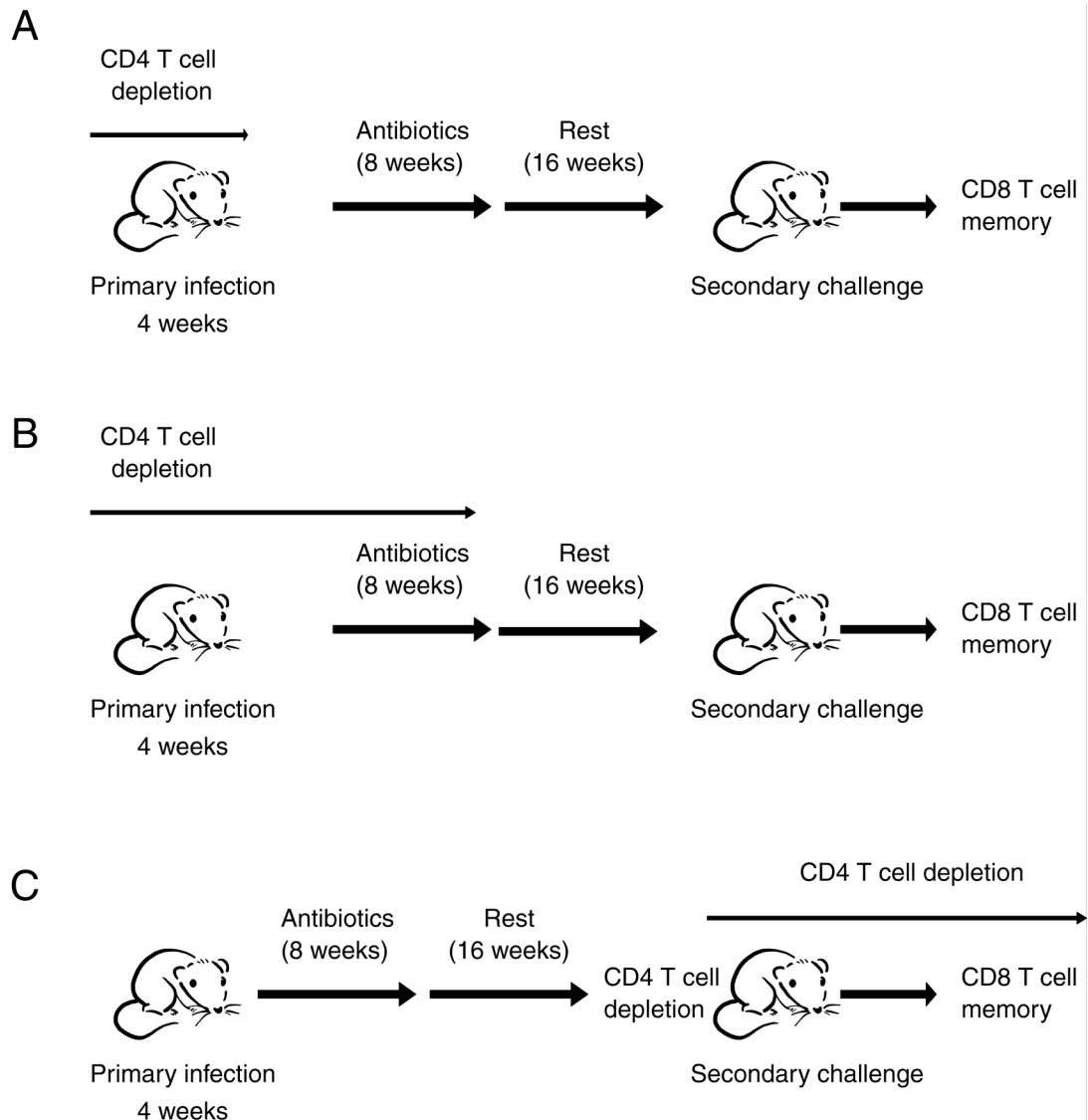


Figure 30 Development of memory models

(A) CD4⁺ T cells were depleted using anti-CD4 (GK1.5) antibody one week prior *M. tuberculosis* infection. Antibody administration was continued twice weekly for 4 weeks during priming of effector CD8⁺ T cell responses. Antibiotic treatment with isoniazid and pyrazinamide began at 4 weeks post-infection and continued for 8 weeks. Mice were rested for 16 weeks prior to secondary challenge with *M. tuberculosis*.

(B) Alternatively, CD4⁺ T cells were depleted one week prior *M. tuberculosis* infection, during primary infection (4 weeks) and throughout antibiotic treatment (8 weeks). Mice were challenged with *M. tuberculosis* following 16 weeks of rest.

(C) Mice were infected with *M. tuberculosis* for 4 weeks, treated with antibiotics for 8 weeks and rested for 16 weeks. One week prior to secondary challenge, CD4⁺ T cells were depleted with GK1.5 antibody, and antibody treatment was continued twice a week for duration of the study.

Mice treated with GK1.5 or IgG during the priming phase were equally capable of controlling secondary challenge with *M. tuberculosis*, as both groups of mice harbored similar bacterial burden in the lung and spleen (Figure 31A and 31B).

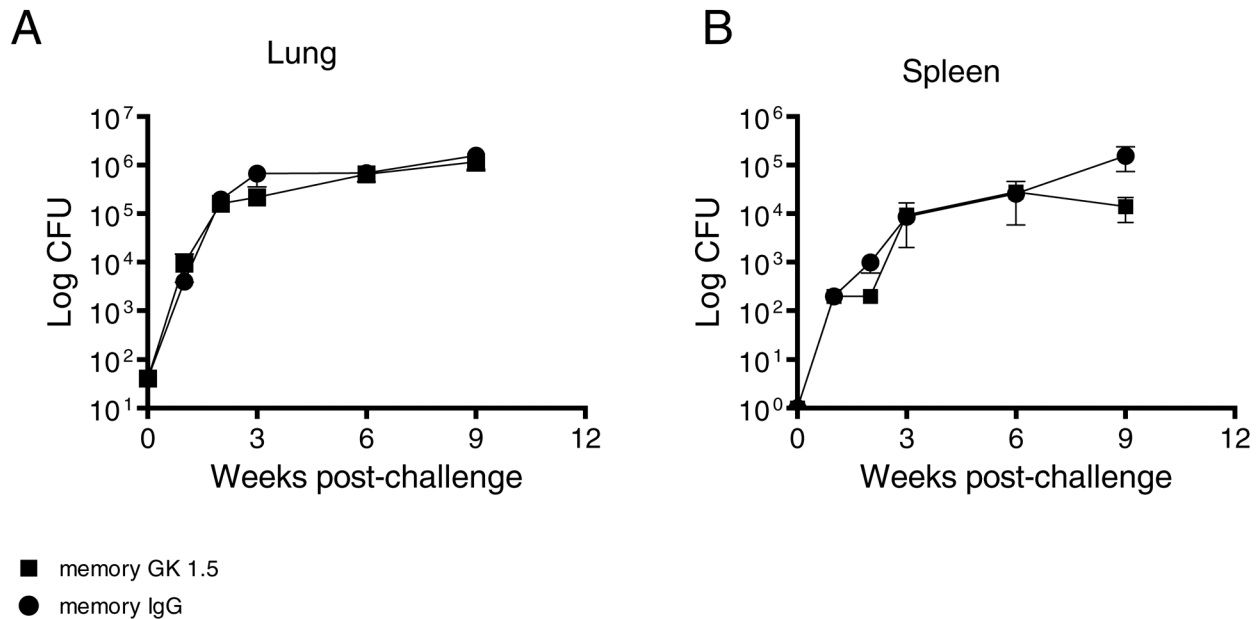


Figure 31 Bacterial burden in the lung and spleen of GK1.5 and IgG treated memory mice

(A-B) Serial dilutions of lung (A) and spleen (B) homogenates were plated on 7H10 plates, and colonies were enumerated after 21 days of incubation. Results indicate that both GK1.5 and IgG treated memory mice were equally capable of controlling *M. tuberculosis* infection. The results represent mean \pm SEM for 4 mice per group.

Early after secondary challenge, IgG treated mice had a significantly higher percentage of CD8⁺ T cells in the lungs (2 weeks post-challenge); nevertheless, by 3 weeks post-challenge both groups of mice had similar percentage of CD8⁺ T cells (Figure 32A). These findings indicate that expansion of memory CD8⁺ T cells was similar in both GK1.5 and IgG treated mice.

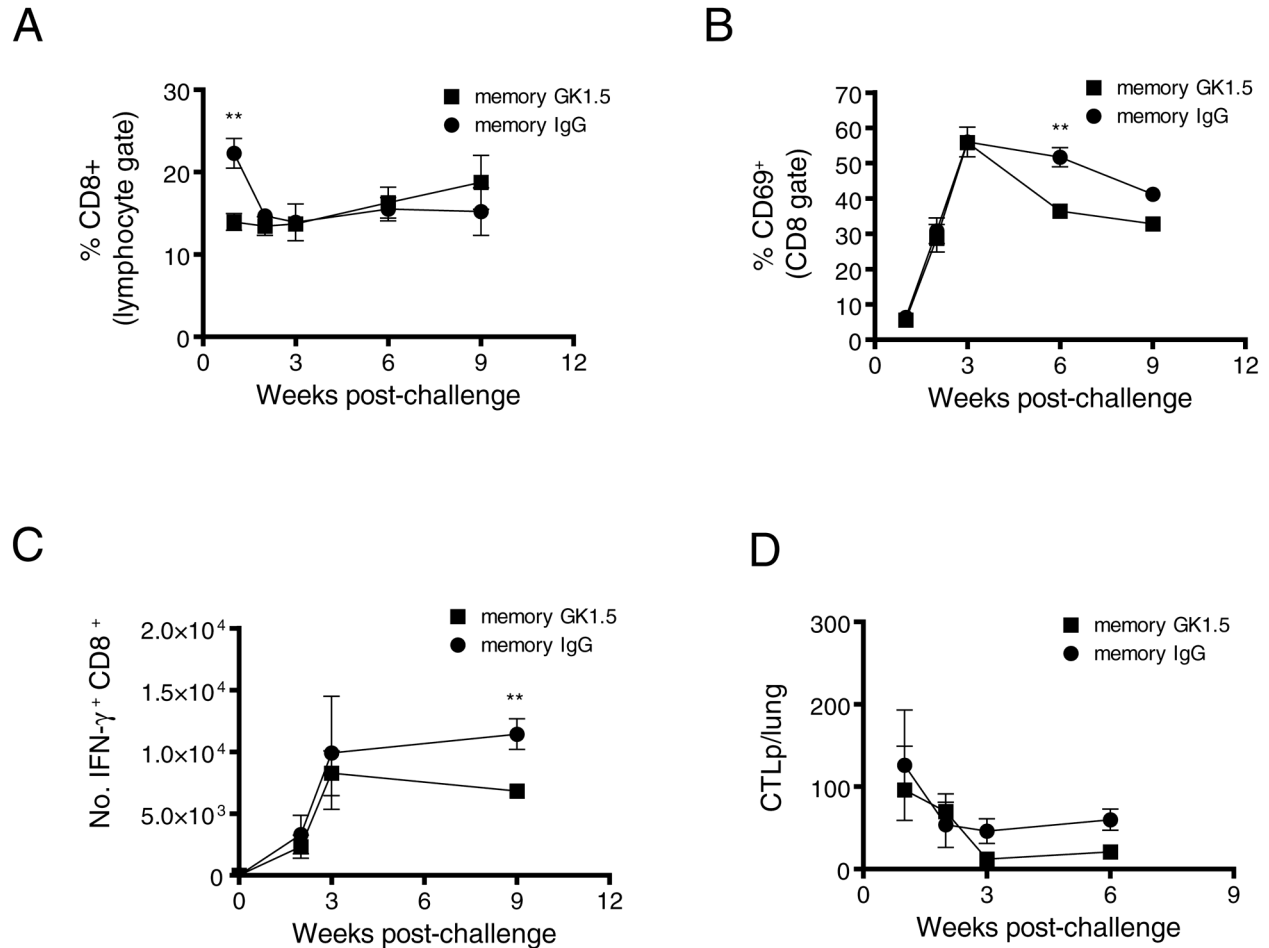


Figure 32 Functional analysis of memory CD8⁺ T cells that were primed in the absence of CD4⁺ T cell help

(A-B) Lung cells were stained with anti-CD8 and anti-CD69 antibodies. The percentage of CD8⁺ T cells within lymphocyte gate (A) and the percentage of CD69⁺ cells within CD8⁺ gate (B) were estimated by flow cytometry.

(C) The number of IFN-γ producing CD8⁺ T cells was determined after *ex vivo* stimulation of lung cells with *M. tuberculosis*-infected dendritic cells pre-incubated with the blocking MHC Class II antibody. Background number of IFN-γ producing T cells after stimulation with uninfected dendritic cells was subtracted before calculations were made. The efficacy of blocking MHC Class II antibody was shown to be >95% at 10 μg/ml concentration. The results in the graphs represent mean ± SEM for 4 mice per group.

(D) The number of CTLp per lung was estimated by limiting dilution analysis as described in Materials and Methods. Serial dilutions of lung cells were subjected to two rounds of stimulation with *M. tuberculosis*-infected dendritic cells, followed by *M. tuberculosis*-infected macrophages. After two weeks of expansion individual wells were tested for cytotoxicity using chromium labeled, *M. tuberculosis*-infected macrophages. The results in the graphs represent mean ± SEM for 4 mice per group.

Initially memory CD8⁺ T cells from both groups of mice responded to the secondary challenge with *M. tuberculosis* with similar kinetics (Figure 32B – 32D). However, as the infection progressed, the function of memory CD8⁺ T cells from the GK1.5 treated mice declined at a faster rate than in control mice (Figure 32B – 32D). There were fewer activated memory CD8⁺ T cells (Figure 32B), and the number of IFN- γ producing CD8⁺ T cells was significantly lower in the lungs of GK1.5 treated mice (Figure 32C). While there was some residual cytotoxic activity in the lungs of IgG treated mice at 6 weeks post-challenge, the number of CTLp in the lungs of GK1.5 treated mice was negligible by 3 weeks post-challenge (Figure 32D).

Functional analyses of CD4⁺ T cells from GK1.5 treated mice revealed that these cells were not truly naïve at the time of secondary challenge. Most likely, CD4⁺ T cells repopulated GK1.5 treated mice before all of *M. tuberculosis* was eliminated by the antibiotic treatment. Subsequently, some of CD8⁺ T cell responses could have been primed in the presence of CD4⁺ T cells later in infection. We attempted to address this problem by depleting CD4⁺ T cells one week prior to infection, during priming of CD8⁺ T cell responses (4 weeks), and throughout antibiotic treatment (8 weeks) (Figure 30B). In this case it was expected that CD4⁺ T cells would repopulate the GK1.5 treated mice after all of the bacteria were cleared by the antibiotics. Surprisingly, mice in which CD4⁺ T cells were depleted for 13 weeks were unable to completely eliminate *M. tuberculosis* despite 2 months of antibiotic treatment (data not shown). In contrast, no viable *M. tuberculosis* was recovered from the lungs and spleens of IgG treated mice. These findings suggest that a 2 month antibiotic regimen is not sufficient to clear *M. tuberculosis* in the face of immunodeficiency, such as lack of CD4⁺ T cells. Due to the presence of residual mycobacteria in CD4⁺ T cell-depleted mice, this study was not included in the analysis of memory CD8⁺ T cell responses.

Although some CD8⁺ T cells may have been primed in the presence of CD4⁺ T cells past 4 weeks post-infection, depletion of CD4⁺ T cells during this initial stage in infection had a significant effect on the function by memory CD8⁺ T cells during secondary challenge.

7.3.2. Effects of CD4 depletion during secondary challenge on the maintenance of memory CD8⁺ T cell responses

Next we asked whether CD4⁺ T cells were required during secondary challenge for the maintenance of functional memory CD8⁺ T cell responses. In the second memory model (Figure 30C), mice were infected with *M. tuberculosis* for the first four weeks of infection, antibiotic treatment began at four weeks for the duration of 8 weeks, followed by 4 months of rest. One week before secondary challenge, CD4⁺ T cells were depleted with GK1.5 antibody, and the depletion regimen continued twice a week for duration of the study. Naïve CD4^{-/-} and wild-type mice were infected as additional controls.

In the absence of CD4⁺ T cells, both naïve and memory mice were unable to control *M. tuberculosis* infection. CD4⁺ T cell-deficient mice had significantly higher bacterial numbers in the lung and spleen when compared to immunocompetent mice (Figure 33A and 33B), and succumbed to *M. tuberculosis* infection at 12 weeks post-infection/challenge (data not shown). For this reason, all functional analyses of effector and memory CD8⁺ T cells were completed by 9 weeks post-infection/challenge.

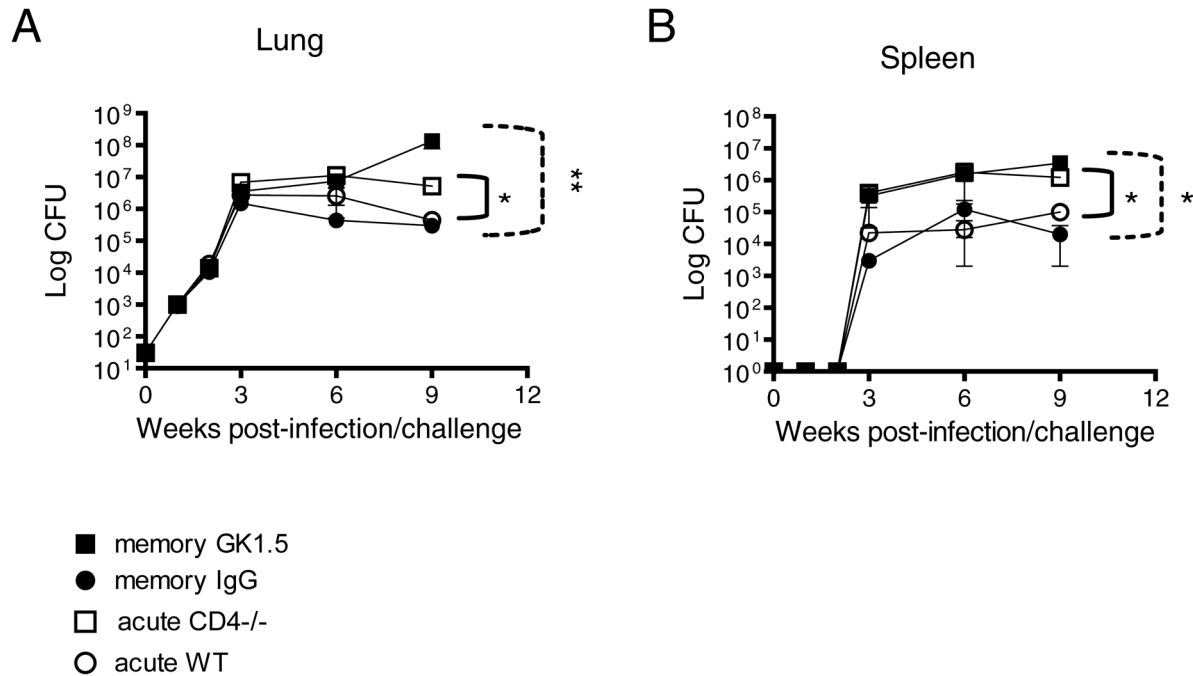


Figure 33 Bacterial burden in the lung and spleen of GK1.5 and IgG treated memory mice, and naïve CD4^{-/-} and wild-type mice

(A-B) Serial dilutions of lung (A) and spleen (B) homogenates from GK1.5 and IgG treated mice as well as from naïve CD4^{-/-} and wild-type mice were plated on 7H10 plates, and colonies were counted following 21 days of incubation. CD4⁺ T cell-deficient memory and naïve mice were unable to contain *M. tuberculosis* infection, and had significantly higher bacterial burden by 9 weeks post-infection. CD4⁺ T cell-deficient memory and naïve mice succumbed to *M. tuberculosis* by 12 weeks post-infection (data not shown). The results are representative of three independent experiments.

7.3.3. Expansion and activation of effector and memory CD8⁺ T cells were unimpaired in the absence of CD4⁺ T cells

There was an increase in the numbers of CD8⁺ T cells in the lungs of GK1.5 memory mice (Figure 34A). Early after secondary challenge both GK1.5 and IgG memory CD8⁺ T cells exhibited increased expression of CD69 when compared to naïve CD4^{-/-} and wild-type mice (Figure 34B). By 3 weeks post-infection a similar percentage of activated CD8⁺ T cells was detected in the lungs of all groups of mice.

Memory CD8⁺ T cells from the lungs of GK1.5 treated mice proliferated significantly more at 3 and 6 weeks post-challenge when compared to IgG treated mice. No significant differences were observed in the percentages of proliferating CD8⁺ T cells from the lungs of naïve CD4^{-/-} and wild-type mice or from the lymph nodes of naïve and memory mice (Figure 34D – 34F).

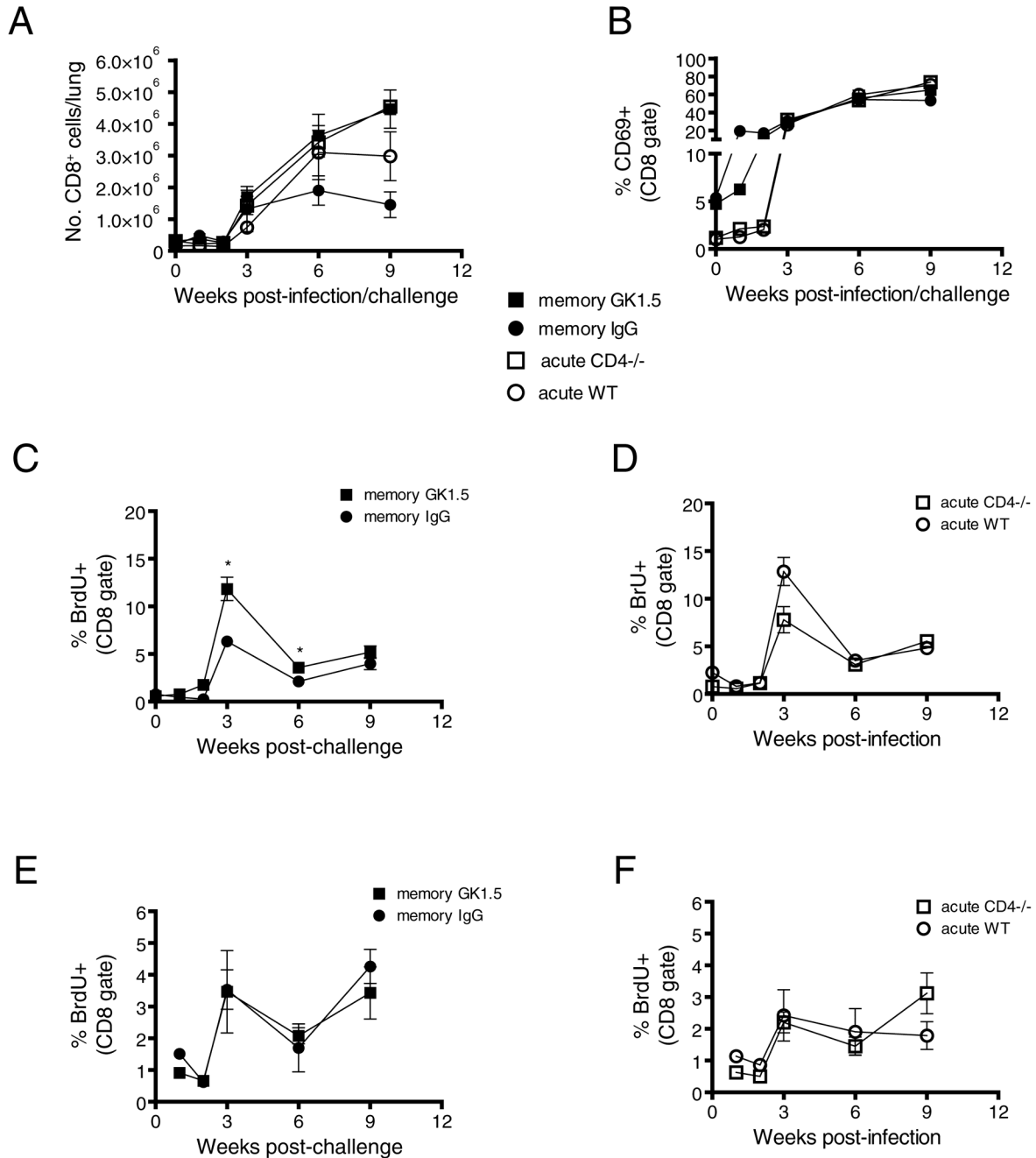


Figure 34 Expansion and activation of memory CD8⁺ T cells in GK1.5 treated mice were unimpaired

(A-B) Lung cells from memory GK1.5 and IgG treated mice and from naïve CD4^{-/-} and wild-type mice were stained with anti-CD8 and anti-CD69 antibodies at designated time points. The number of CD8⁺ T cells within live cell gate (A), and the percentage of CD69⁺ T cells within CD8⁺ gate (B) were estimated by flow cytometry.

(C-D) The percentage of proliferating cells was estimated by flow cytometry after 16 hour injection of BrdU intraperitoneally. The percentage of proliferating cells within CD8⁺ gate was determined for the lung samples from memory (C) and acutely infected mice (D). The data represent mean ± SEM for 3 mice per group.

(E-F) The percentage of proliferating cells within CD8⁺ gate in the lymph nodes of memory (E) and naïve (F) mice was determined by BrdU incorporation. The data represent mean ± SEM for 3 mice per group.

7.3.4. Phenotypic characterization of memory CD8⁺ T cells

Two distinct subsets of memory CD8⁺ T cells have been described, namely “central”/“resting” memory CD8⁺ T cells (T_{CM}) and effector memory CD8⁺ T cells (T_{EM}). These two subsets are differentiated by their migratory properties, functions, and expression of lymph node-homing molecules CD62L and CCR7 (304-307). T_{CM} express CD62L and CCR7, while T_{EM} are CD62L⁻ and CCR7⁻ (304). Murine memory CD8⁺ T cells are also characterized as being CD44^{hi}, Ly6C^{hi} and CD69⁻ (308). In this study “resting” memory cells were defined as CD8⁺ Ly6C^{hi} CD62L⁺ or CD8⁺ Ly6C^{hi} CD69⁻; and “effector” memory cells were described as being CD8⁺ Ly6C^{hi} CD62L⁻ or CD8⁺ Ly6C^{hi} CD44^{hi} (Figure 35). We found that the proportion of “resting” CD8⁺ T cells decreased following *M. tuberculosis* infection in the lungs and lymph nodes of all mice (Figure 35A and 35B). However, there was no correlation in the relative presence of “resting” memory CD8⁺ T cells between naïve and immune mice even before primary infection or secondary challenge. In fact, according to the flow cytometric analysis, naïve mice had a higher percentage of “memory”-like cells than immune mice (Figure 35A and 35B). Initially the percentage of “effector” memory CD8⁺ T cells was higher in the lungs of IgG treated memory mice after secondary challenge (Figure 35C). Later in infection both memory and naïve mice had a similar percentage of “effector” memory cells in the lungs, while there was no pattern in the expression of these memory markers in the lymph nodes (Figure 35C and 35D). These data indicate that a very heterogenous population of CD8⁺ T cells exists in the lungs following primary and secondary infection with *M. tuberculosis*. Although these cell surface markers clearly delineate memory CD8⁺ T cells in mice following viral infections, we found that neither combination of the cell surface markers corresponded to the functional characteristics of

memory CD8⁺ T cells in murine model of tuberculosis, most likely due to the chronic nature of the infection.

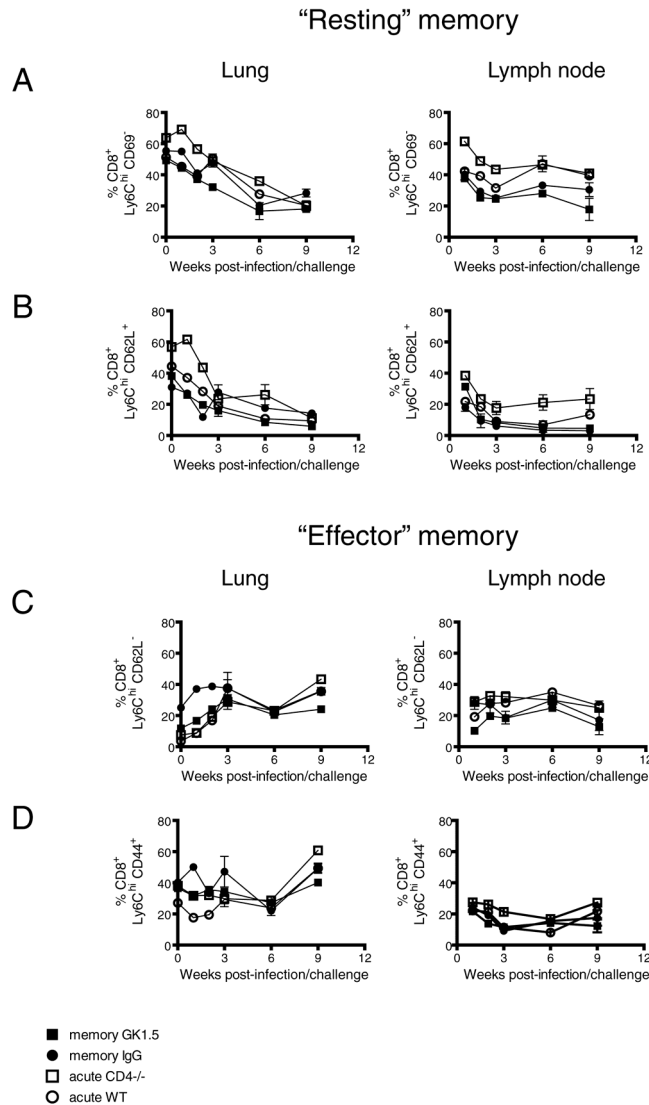


Figure 35 Phenotypic characterization of memory CD8⁺ T cells from GK1.5 and IgG treated memory mice

(A-B) The “resting”/“central” memory cells were defined as CD8⁺ Ly6C^{hi} CD69⁻ (A) or CD8⁺ Ly6C^{hi} CD62L⁺ (B). The percentage of “resting” memory CD8⁺ T cells was determined for the lungs and lymph nodes of memory GK1.5 and IgG treated mice. Lung and lymph nodes of naïve CD4^{-/-} and wild-type mice were also stained and analyzed for the presence of “resting” memory cells as additional controls.

(C-D) The “effector” memory cells were described as CD8⁺ Ly6C^{hi} CD62L⁻ (C) or CD8⁺ Ly6C^{hi} CD44^{hi} (D). The percentage of “effector” memory cells was determined for the lung and lymph node samples from GK1.5 and IgG treated memory mice as well as naïve CD4^{-/-} and wild-type mice. The results represent mean ± SEM for 3 mice per group.

7.3.5. IFN- γ producing CD8⁺ T cells cannot persist without CD4⁺ T cell help

The number of IFN- γ producing CD8⁺ T cells was determined after *ex vivo* stimulation of lung cells with *M. tuberculosis*-infected dendritic cells pre-incubated with the blocking MHC Class II antibody. The number of IFN- γ producing *M. tuberculosis*-specific CD8⁺ T cells did not expand and declined in the lungs and the lymph nodes of memory mice in absence of CD4⁺ T cells (Figure 36A and 36C). In contrast, the number of IFN- γ producing CD8⁺ T cells from IgG treated memory mice continued to rise as infection progressed (Figure 36A and 36C). Although similar numbers of CD8⁺ effector cells from naïve CD4^{-/-} mice produced IFN- γ during acute infection (up to 3 weeks), the magnitude of IFN- γ ⁺ CD8⁺ T cell responses was significantly lower than in wild-type mice during chronic infection (9 weeks) (Figure 36B and 36D).

The number of CTLp in the lungs of GK1.5 and IgG treated memory mice was similar suggesting that the absence of CD4⁺ T cells did not have a significant impact on the development and maintenance of memory cytotoxic CD8⁺ T cell responses. No CTL activity was detected in any of the groups of mice at 9 weeks post-challenge (data not shown).

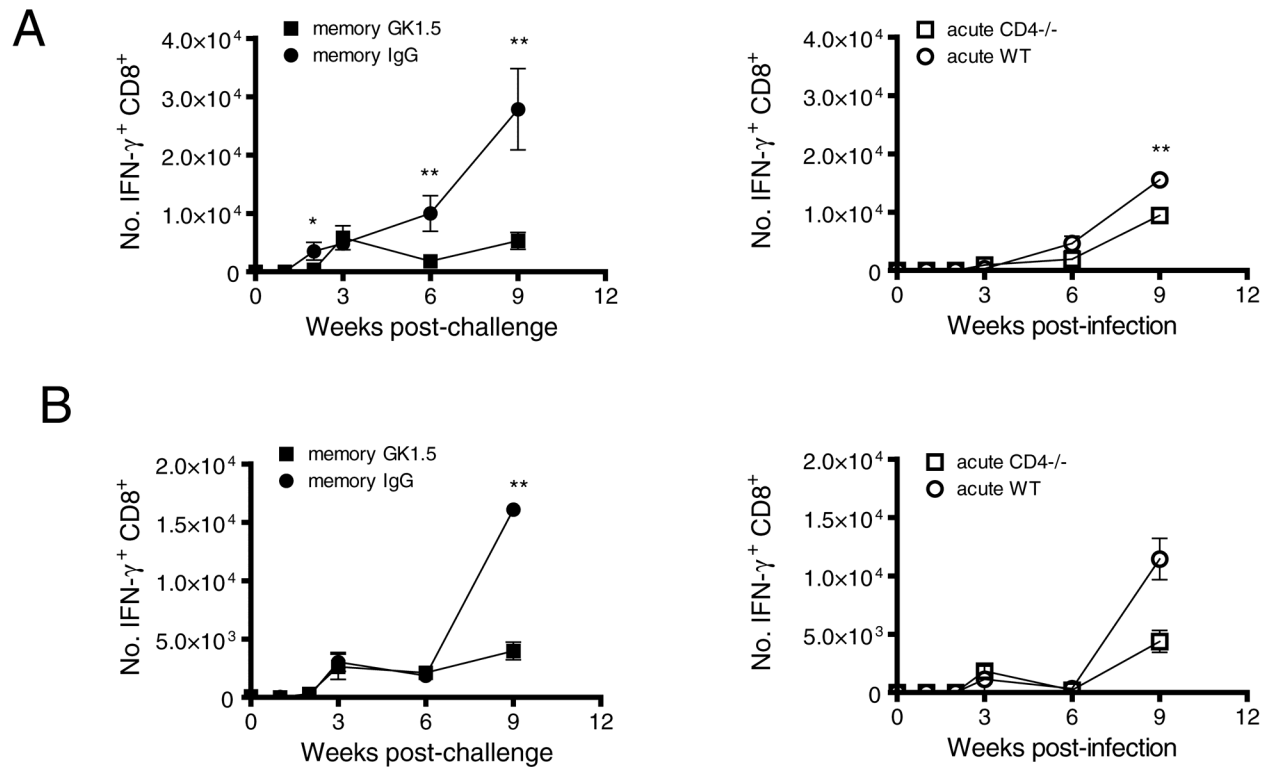


Figure 36 Loss of IFN- γ producing CD8⁺ T cells in the lungs and lymph nodes of memory mice without CD4⁺ T cell help

(A-B) The number of IFN- γ producing CD8⁺ T cells in the lungs (A) and lymph nodes (B) of memory and naïve mice was determined by ELISPOT as described in Materials and Methods. The results represent the mean of 2 – 6 mice per group.

7.4. Discussion

In this study we investigated the requirement for CD4⁺ T cells during primary infection or secondary challenge for the generation and maintenance of memory CD8⁺ T cell responses in *M. tuberculosis* infection. Depletion of CD4⁺ T cells during primary infection resulted in generation of memory CD8⁺ T cells that responded with equal kinetics as memory CD8⁺ T cells from control mice; however as infection progressed the frequency of activated, cytotoxic and cytokine producing memory CD8⁺ T cells significantly declined in mice that did not receive CD4⁺ T cell help during priming. Depletion of CD4⁺ T cells during secondary challenge resulted in generation of memory CD8⁺ T cells that responded with similar kinetics to the initial challenge with *M. tuberculosis*; however as infection progressed, memory CD8⁺ T cells without CD4⁺ T cell help never reached the same magnitude as “helped” memory CD8⁺ T cells. These findings suggest that CD4⁺ T cell help is required for the generation of long-lasting and functional memory CD8⁺ T cells. Unlike other studies, which reported a major defect in the proliferation of memory CD8⁺ T cells without CD4⁺ T cell help (299-301), we found no evidence of impaired expansion in “unhelped” memory CD8⁺ T cells. The most striking difference between “helped” and “unhelped” memory CD8⁺ T cells was in the durability and magnitude of their effector functions during the recall response against *M. tuberculosis*.

Previous work from our laboratory demonstrated that memory CD8⁺ T cells participated in the recall responses against *M. tuberculosis* (302). Since development of cytotoxic CD8⁺ T cell responses depended on CD4⁺ T cells during acute *M. tuberculosis* infection (138), we next investigated whether CD4⁺ T cell help was also required for the generation and maintenance of memory CD8⁺ T cells. To delineate when CD4⁺ T cell help may be required for the development of memory CD8⁺ T cells, we designed two memory models. In the first memory model, we

depleted CD4⁺ T cells during primary infection and asked whether CD4⁺ T cells were required during priming of effector CD8⁺ T cells for the subsequent generation of memory CD8⁺ T cells. Although the percentage of memory CD8⁺ T cells in both groups of mice was similar, activation and IFN- γ production by memory CD8⁺ T cells from GK1.5 treated mice significantly declined following secondary challenge. The difference between “helped” and “unhelped” memory CD8⁺ T cell responses in this model may be underestimated by the fact that during the secondary challenge CD4⁺ T cells did not exhibit a truly naïve phenotype. These findings suggest that some proportion of CD8⁺ T cells may have received CD4⁺ T cell help past the initial four weeks of priming. We attempted to address this issue by prolonging the CD4 depletion from 4 weeks to 12 weeks post-infection. Although this antibiotic regimen (isoniazid and pyrazinamide for 8 weeks) showed sterilizing activity in latency reactivation studies (47), surprisingly, CD4 depleted mice were unable to completely clear *M. tuberculosis* infection. These CD4 depleted mice harbored 1×10^5 CFU in the lungs and 1×10^4 CFU in the spleen at the time of secondary challenge; therefore excluding this study as a model of CD8⁺ T cell memory. Since antibiotics were insufficient at eliminating *M. tuberculosis*, these results underscore the importance of CD4⁺ T cell mediated protective responses against *M. tuberculosis* even during optimal chemotherapy. Nevertheless, our data indicate that the absence of CD4⁺ T cells during the first four weeks of infection resulted in a significant reduction of IFN- γ producing CD8⁺ T cells during secondary challenge.

In the second memory model, we depleted CD4⁺ T cells during secondary challenge and studied the effects of CD4⁺ T cells on the maintenance of CD8⁺ T cell responses. Memory CD8⁺ T cells from GK1.5-treated mice showed no defects in proliferation as measured by BrdU incorporation and had a similar percentage of activated CD69⁺ CD8⁺ T cells as IgG treated mice

throughout the secondary challenge. However, IFN- γ producing CD8⁺ T cells did not expand substantially in the lungs of GK1.5-treated mice, in contrast to control mice. These results demonstrate a dependence on CD4⁺ T cells for optimal CD8⁺ T cell function and maintenance at both the priming and restimulation (challenge) phases of infection with *M. tuberculosis*. In the absence of CD4⁺ T cell help, IFN- γ producing CD8⁺ T cells perished as infection progressed to 9 weeks post-challenge. Even acutely infected CD4^{-/-} mice showed dependence on CD4⁺ T cell help in the maintenance of IFN- γ producing CD8⁺ T cells during the chronic stage of infection. It appears that effector CD8⁺ T cells are more dependent on CD4⁺ T cells help in the context of antigen specific stimulation than previously shown with non-specific stimulation with anti-CD3/CD28 antibodies (138).

Phenotypic characterization of memory CD8⁺ T cells in naïve and immune CD4⁺ T cell-deficient and wild-type mice did not yield conclusive results in this model. In this study, we defined “central”/“resting” memory CD8⁺ T cells as CD8⁺ Ly6C^{hi} CD69⁻ or CD8⁺ Ly6C^{hi} CD62L⁺ and “effector” memory CD8⁺ T cells as CD8⁺ Ly6C^{hi} CD62L⁻ or CD8⁺ Ly6C^{hi} CD44^{hi}. Our analysis mainly focused on the relative expression of Ly6C as almost all CD4⁺ and CD8⁺ T cells in the lungs of *M. tuberculosis* infected lungs are CD44^{hi}. Expression of CD62L and the lack of CD69 marker on CD8⁺ Ly6C^{hi} cells were the main criteria for defining cells as being “resting”, while lack of CD62L expression and high expression of CD44 on CD8⁺ Ly6C^{hi} cells identified cells as being of “effector memory” phenotype. The baseline staining of uninfected naïve mice, and unchallenged immune mice yielded confusing results as naïve mice had a higher percentage of “memory”-like cells in the lungs and the lymph nodes than memory mice. Thus, the use of standard markers for murine memory CD8⁺ T cells did not represent the functional characteristics of memory CD8⁺ T cells in murine model of tuberculosis. This may be due in part

to the lack of tetramer reagents for identifying specific T cells in the lungs, but our previous data suggest that most of the CD8⁺ T cells in the lungs following *M. tuberculosis* infection are specific for mycobacterial antigens. Hence, the use of these cell surface markers for representation of *M. tuberculosis*-specific memory CD8⁺ T cells should be interpreted with caution.

The mechanisms of CD4⁺ help in the generation of effector and memory CD8⁺ T cell responses during *M. tuberculosis* infection were extensively investigated in our laboratory. A similar percentage of CD8⁺ T cells expressed perforin in the lungs and lymph nodes of CD4⁺ T cell-deficient and wild-type mice suggesting that CD8⁺ T cells with or without CD4⁺ T cell help were equipped with cytotoxic arsenal (data not shown). However, CD8⁺ T cells from CD4⁺ T cell-deficient mice failed to employ their cytotoxic effector mechanisms (138). Although CD4⁺-T cell deficient mice had significantly less IL-2 and IL-15 in the lungs following acute *M. tuberculosis* infection (138), effector and memory CD8⁺ T cell responses developed normally in IL-15^{-/-} mice (Chapter 3). Our previous studies demonstrated that CD40^{-/-} mice were very susceptible to *M. tuberculosis* infection as a result of reduced priming of IFN- γ T cell responses (228). In fact, CD4⁺ T cells were more affected by the absence of the CD40 molecule, while no significant differences were detected in the numbers of IFN- γ producing CD8⁺ T cells between CD40^{-/-} and wild-type mice (228). The cytotoxic activity of CD8⁺ T cells in CD40^{-/-} and CD40L^{-/-} mice was comparable to wild-type mice (data not shown). Collectively, these results indicate that CD40/CD40 co-stimulation and the availability of IL-15 are not the primary helper mechanisms of CD4⁺ T cells in *M. tuberculosis* infection model.

Since the primary goal of vaccines against tuberculosis is to elicit long-term protective immunity, it is essential to understand which factors regulate development and maintenance of T

cell memory. In this study, we have investigated the importance of CD4⁺ T cells in the generation of effective memory CD8⁺ T cell responses. Our data show that CD4⁺ T cells are required both during priming of effector CD8⁺ T cells and during secondary challenge for the long-term maintenance of memory IFN- γ ⁺ CD8⁺ T cells.

8. SUMMARY OF THE THESIS

Mycobacterium tuberculosis infection is acquired through inhalation of aerosolized droplets containing the bacilli generated by a person with active disease. In the case of productive infection, *M. tuberculosis* infects resident alveolar macrophages and dendritic cells. Infected macrophages set off the inflammatory cascade by secreting proinflammatory cytokines, resulting in the recruitment of more immune cells into the lungs. Dendritic cells mature upon *M. tuberculosis* infection and migrate to the lung draining lymph nodes where they prime naïve T cells. Activated T cells acquire their effector functions, such as cytokine production and cytotoxicity, and infiltrate the infected lung where they recognize *M. tuberculosis* infected macrophages. The net result is formation of granulomas where infected macrophages are surrounded by newly recruited macrophages, T and B lymphocytes. The main function of granulomas structure is to seal off the foci of infection, prevent dissemination of *M. tuberculosis*, and allow for macrophage – T cell contacts leading to the induction of mycobactericidal mechanisms in infected macrophages and control of *M. tuberculosis* replication.

Although we have come a long way in understanding the series of events that lead to protection against *M. tuberculosis*-induced disease, still very little is known about what conditions are required for the induction of protective T cell mediated immunity. Furthermore, we do not understand how persistent stimulation of CD4⁺ and CD8⁺ T cells with *M. tuberculosis* antigens affects their function, and since the goal of all anti-tuberculosis vaccines is to elicit long-lasting and protective immunity, we definitely need to identify factors that are required for the development and maintenance of functional memory T cell responses.

The studies presented in this thesis attempt to answer these questions. In Chapter 1 we provide evidence for the importance of the CD40 molecule in the induction of protective IFN- γ T

cell responses. The results in Chapter 2 demonstrate that once a successful immune response is initiated, the long-term control of *M. tuberculosis* is mediated by dynamic changes in the frequency and effector functions of CD4⁺ and CD8⁺ T cells in a setting that only appears to be a steady balance between the pathogen and the host's immune response. In Chapter 3, we addressed the role of IL-15 in the maintenance of T cell effector functions, and also in the generation of CD4⁺ and CD8⁺ T cell memory, while in Chapter 4 we investigated the requirement for CD4 helper mechanisms in the development and maintenance of memory CD8⁺ T cell responses.

Our results indicate that CD40 ligation on dendritic cells was necessary for the initiation of robust T cell mediated immunity and containment of *M. tuberculosis* infection, while the absence of CD40L did not have a substantial effect on the development of T cell responses (Chapter 1). Work with CD40^{-/-} mice demonstrated that in the absence of CD40 signaling, IL-12 production by dendritic cells was minimal leading to the poor priming of IFN- γ of T cell responses. Consequently, infiltration of effector cells into infected lungs of CD40^{-/-} mice was significantly diminished when compared to wild-type mice leading to the susceptibility of CD40^{-/-} mice to *M. tuberculosis* infection. Exogenous administration of IL-12 to *in vitro* CD40^{-/-} dendritic cell – T cell co-cultures, or injection of IL-12 during priming of T cell responses significantly enhanced the priming ability of CD40^{-/-} dendritic cells and resulted in the generation of strong, robust IFN- γ T cell responses. Dependence on CD40 ligation for induction of protective IFN- γ T cell responses was a function of antigen dose. Priming of IFN- γ T cell responses was dependent on CD40 mediated signals after low dose aerosol infection with *M. tuberculosis*. However, in the case of systemic infection or infection with a higher antigen dose, induction of IFN- γ T cell responses was CD40 independent possibly due to signaling through

Toll-like receptors. Based on these results, we created a following model that describes a series of events that will lead to the priming of strong Th1 response. *M. tuberculosis* infection of dendritic cells results in phenotypic changes that are indicative of maturation characterized by upregulation of antigen presenting and costimulatory molecules. Although we have no evidence that dendritic cells that were infiltrating the lung draining lymph nodes were directly infected with *M. tuberculosis*, we detected a significant increase in their numbers which coincided with the priming of T cell responses. In the lymph nodes, CD40 ligation on dendritic cells, either by CD40L on CD4⁺ T cells and/or by *M. tuberculosis*-derived components, such as Hsp70, induces optimal IL-12 production by dendritic cells. High levels of IL-12 in the lymph nodes result in the priming of IFN- γ producing T cells that leave the lymph nodes and infiltrate infected lungs conferring protection against *M. tuberculosis*.

During initial stage of infection CD4⁺ T cells are the primary source of IFN- γ , while contribution of CD8⁺ T cells to the IFN- γ production is existent but minimal until chronic stage of infection, when the frequency of IFN- γ producing CD8⁺ T cells is equivalent to IFN- γ producing CD4⁺ T cells (Chapter 2). CD8⁺ T cells respond to *M. tuberculosis* infection by employing preferentially the cytotoxic mechanisms during acute infection, and switch to cytokine production during chronic infection. This switch will activate RNI and ROS pathways in infected macrophages leading to the destruction of intracellular bacteria, thereby minimizing immunopathology that would otherwise be invoked by extensive cytotoxic activity during infection. Nevertheless, CD8⁺ T cells retained the ability to synthesize perforin, which was detected by both intracellular perforin staining and immunohistochemistry. These results suggest that CD8⁺ T cells were not exhausted during chronic infection, but restrained from employing their cytotoxic activity (Chapter 2). One of the most important candidates for determining the

development fate of CD8⁺ T cells is antigen dose. Several studies have reported that at low levels of TCR occupancy an immature synapse is formed, which is sufficient to trigger cytotoxicity, but not cytokine production (245, 246). In contrast, the high level of TCR occupancy is required to stimulate cytokine production and proliferation (245, 246). In our study we were unable to correlate CD8⁺ CTLp frequency with the total number of viable bacteria in the lungs. However, determination of CFUs is a crude estimate of antigenic load that CD8⁺ T cells may be exposed to *in vivo* as it measures only the number of replication-competent mycobacteria. It is very difficult to estimate the epitope density on the surface of infected macrophages. Russell *et al.* reported that mycobacterial antigens, particularly lipids and glycolipids, traffic dynamically to the cell surface of infected cells (251-253). Thus, the physiological relevance of antigen dose on effector CD8⁺ T cell functions in *M. tuberculosis* infection remains to be resolved.

A surprising result was that the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells dynamically waxed and waned during persistent *M. tuberculosis* infection (Chapter 2). We propose two explanations for such behavior of effector T cells. It is generally accepted that during persistent infection, *M. tuberculosis* exists in a quiescent state characterized by low level of metabolic activity. Nevertheless, it is possible that *M. tuberculosis* transiently undergoes bursts of replication. These changes in bacterial numbers are sensed by the host immune system, which responds by rapidly increasing the numbers of IFN- γ producing T cells until bacterial growth is brought under control. In this manner, bacterial growth will be strictly controlled, with minimal immunopathology that otherwise would be induced by persistently elevated levels of inflammatory cytokines, such as IFN- γ . This explanation suggests a dynamic equilibrium achieved between the pathogen and the host's immune system that persists throughout the course of infection. Alternatively, T cells may be rendered transiently anergic and unable to respond to

constant antigenic stimulation, which would be characterized by drops in the frequency of IFN- γ producing T cells. The regular periodicity of the peaks and troughs in the frequency of IFN- γ producing T cells suggests that a certain time interval is required for T cells to renew their IFN- γ producing capacity. A detailed investigation of TCR signaling at different time points during chronic infection will resolve whether transient anergy in T cell activation exists due to persistent exposure to mycobacterial antigens. Nevertheless, the T cell IFN- γ production is not permanently silenced by high antigen doses, suggesting that the effects of persistent mycobacterial antigenic stimulation on T cell functionality are transient. Unlike many chronic viral infections where T cells undergo rapid turnover, and enter the state of replicative senescence, there is no evidence that this is the case during persistent *M. tuberculosis* infection as CD4⁺ and CD8⁺ T cells underwent two bursts of replication during 7 months of infection (Chapter 2).

Work with IL-15^{-/-} mice demonstrated that this cytokine is not essential for the generation and maintenance of primary CD4⁺ and CD8⁺ T cell responses (Chapter 3). Contrary to acute virus infections where IL-15 was shown to be important for the expansion of virus-specific primary or memory CD8⁺ T cells (263, 264), the absence of IL-15 did not affect the proliferation or cytokine production by CD4⁺ and CD8⁺ T cells. Functional cytotoxic CD8⁺ T cells were also detected in IL-15^{-/-} mice. These findings suggest that IL-15 is not essential for the priming of IFN- γ producing CD4⁺ and CD8⁺ T cells or cytotoxic CD8⁺ T cells. Moreover, proliferation and the long-term maintenance of CD4⁺ and CD8⁺ T cell effector functions were not dependent on the presence of IL-15. Our data indicate that IL-15 was not required for the proliferative renewal of memory *M. tuberculosis*-specific T cells or for the maintenance of the memory responses against *M. tuberculosis*. Therefore, infections with viral and bacterial

pathogens may result in different requirements for the generation and maintenance of primary and memory T cell responses.

However, the findings from Chapter 4 demonstrate that development and functionality of memory CD8⁺ T cell responses were dependent of CD4 helper mechanisms. Depletion of CD4⁺ T cells during priming of primary CD8⁺ T cell responses or during secondary challenge resulted in low magnitude recall responses by memory CD8⁺ T cells. Depletion of CD4⁺ T cells during primary infection resulted in generation of memory CD8⁺ T cells that responded with the equal kinetics as memory CD8⁺ T cells from control mice; however as infection progressed the frequency of activated, cytotoxic and cytokine producing memory CD8⁺ T cells significantly declined in mice that did not receive CD4 help during priming. Depletion of CD4⁺ T cells during secondary challenge resulted in generation of memory CD8⁺ T cells that responded with similar kinetics to the initial challenge with *M. tuberculosis*; however as infection progressed, memory CD8⁺ T cells without CD4⁺ T cell help never reached the same magnitude as “helped” memory CD8⁺ T cells. These findings suggest that CD4⁺ T cell help is required for the generation of long-lasting and functional memory CD8⁺ T cells.

In conclusion, this thesis has addressed several important issues of T cell biology during *M. tuberculosis* infection, namely priming, maintenance of effector functions and development of functional memory T cell responses. We found that CD40 ligation on dendritic cells by host- or mycobacterial-derived ligands was essential for induction of optimal IL-12 production and priming of IFN- γ producing T cells. In contrast, we found no evidence that IL-15 was required for the priming of IFN- γ producing or cytotoxic T cells, suggesting that this cytokine is not pivotal for the initiation of potent Th1 response. Once effector T cells were successfully primed, they infiltrated infected lungs where they elaborated their effector functions. Our results indicate

that CD4⁺ T cells responded by IFN- γ production, while CD8⁺ T cells were preferentially cytotoxic and killed infected target cells. As infection was brought under control, CD8⁺ T cells silenced their cytolytic activity and switched to cytokine production. Long-term control of *M. tuberculosis*-infection was characterized by dynamic changes in the frequency of IFN- γ producing CD4⁺ and CD8⁺ T cells. IL-15 was not required for the long-term maintenance of CD4⁺ and CD8⁺ T cell effector functions. In addition, we found no evidence that IL-15 was involved in the development of functional T cell memory. However, development of memory CD8⁺ T cells was dependent of CD4⁺ T cell helper mechanisms.

The findings in this thesis broadened our knowledge of what is required to establish protective immunity against *M. tuberculosis*, and improved our understanding of what is needed to generate functional memory CD8⁺ T cell responses. However, this work has not revealed what the helper mechanisms of CD4⁺ T cells are for the normal functionality of primary and memory CD8⁺ T cells responses. Future work should be directed towards identification of the CD4⁺ T cell helper mechanisms, so that in the case of CD4⁺ T cell deficiency caused by HIV infection, we could provide the helper signals to CD8⁺ T cells through immunotherapy, and enhance their ability to contain *M. tuberculosis* infection. Furthermore, these findings will also enhance the efficacy of newly designed vaccines that target CD8⁺ T cell responses.

APPENDIX A

IL-15 administration did not increase the survival of susceptible CD4^{-/-} mice following low dose aerosol infection with *M. tuberculosis*

A.1 Introduction

In murine studies, IL-15 transgenic mice exhibited increased resistance to *M. bovis* BCG infection, which in part could be attributed to increased numbers of NK cells and augmented IFN- γ production by CD8⁺ T cells (273). Furthermore, IL-15 improved the efficacy of *M. bovis* BCG vaccine in conferring protection against virulent *M. tuberculosis* H37Rv challenge. BCG-vaccinated IL-15 transgenic mice had significantly lower bacterial burden when compared with BCG-vaccinated wild-type mice (274). The increased protective effect observed in IL-15 transgenic BCG vaccinated mice was accompanied by enhanced IFN- γ CD8⁺ T cell responses (274). IL-15 administration also protected susceptible BALB/c mice against virulent *M. tuberculosis* infection when given as a treatment at 3 weeks post-infection (275). Collectively these findings suggest that IL-15 exogenous administration can enhance protective immunity against *M. tuberculosis* infection by augmenting CD8⁺ T cell responses.

In our initial studies, we have demonstrated that development of cytotoxic CD8⁺ T cells was dependent on CD4⁺ T cell helper mechanisms (138). Furthermore, IL-15 mRNA expression was significantly reduced in the lungs of CD4^{-/-} mice when compared to wild-type mice (138). We postulated that one of the helper mechanisms by which CD4⁺ T cells could affect development and maintenance of CD8⁺ T cell effector functions was by influencing the availability of IL-15 cytokine. Since CD4^{-/-} mice had diminished expression of IL-15, we hypothesized that exogenous administration of IL-15 could restore CD8⁺ T cell effector

functions and increase the survival of CD4^{-/-} mice after low dose aerosol infection with *M. tuberculosis*.

A.2 Materials and Methods

Mice and infections

C57BL/6 wild-type mice were purchased from Charles River Laboratories (Wilmington, MA). CD4^{-/-} (C57BL/6 background) were bred at University of Pittsburgh Biotech Center. All mice were kept under specific pathogen-free conditions in a Biosafety Level 3 facility. Animal protocols used in this study were approved by the University Institutional Animal Care and Use Committee. Mice were infected with a low dose of *M. tuberculosis* (Erdman strain, Trudeau Institute, Saranac Lake, NY) at 5×10^5 /ml using a nose-exposure only aerosolizer unit (Intox Inc., Moriarty, NM). The dose received was estimated by plating whole lung homogenates of two mice 24 hours following aerosol infection (~30 CFU/mouse).

IL-15 administration

CD4^{-/-} and wild-type mice were injected with 1 µg/200 µl of human IL-15 daily starting at 5 days post-infection until 21 days post-infection. Control CD4^{-/-} and wild-type mice were injected with 200 µl PBS.

CFU determination

Lung homogenates were serially diluted in PBS/0.05% Tween-80 and plated on 7H10 agar plates (Difco). Plates were incubated at 37°C, 5% CO₂ for 21 days prior to counting colonies.

Bone marrow derived macrophages and dendritic cell cultures

In *ex vivo* stimulation assays, such as ELISPOT and limiting dilution analysis, bone marrow derived dendritic cells were cultured in the presence of 20 ng/ml of GM-CSF and 20 ng/ml of IL-4 (PeproTech Inc, Rocky Hill, NJ) and macrophages were cultured in the presence of L cell supernatant as a source of CSF-1 using standard procedure described previously (146).

Flow cytometry

Lung single cell suspensions were stained as described previously (146). Cells were stained with anti-CD4 (clone H129.19), anti-CD8 (clone 53-6.7), and anti-CD69 (clone H1.2F3) fluorescently conjugated antibodies. All antibodies were purchased from BD Pharmingen (San Diego, CA) and used at 0.2 µg/ml concentration. Cells were collected on a FACSCaliber (Beckon Dickinson, San Jose, CA) and analyzed by CellQuest (Becton Dickinson) or FlowJo (Tree Star Inc, San Carlos, CA) software.

IFN-γ production

Cytokine production by CD8⁺ T cells isolated from the lungs of *M. tuberculosis* infected mice was evaluated by ELISPOT as described previously (240, 241). Briefly, lung and lymph node cells were plated in anti-IFN-γ antibody (BD Pharmingen, clone R4-6A2) coated plates (MAIPS4510, Millipore Corp, Bedford, MA) at 80,000 cells/well and 150,000 cell/well, respectively. Cells were incubated in duplicate wells with media, ConA (10 µg/ml; Sigma-Aldrich), uninfected and *M. tuberculosis*-infected dendritic cells (MOI 3; overnight) to estimate the number of total number of IFN-γ producing T cell, and *M. tuberculosis*-infected dendritic cells incubated with the blocking anti-MHC Class I (BD Pharmingen, clone 8F12) or anti-MHC

Class II (BD Pharmingen, clone M5/114.15.2) antibodies at 10 µg/ml to estimate the number of IFN-γ producing CD4⁺ and CD8⁺ T cells, respectively. All dendritic cells were added to lung and lymph nodes cells at 1:2 ratio and the cultures were supplemented with IL-2 (PeproTech) at final concentration of 20 U/ml.

Following 40 hour incubation, the IFN-γ producing T cells were visualized after stepwise incubation of plates with biotinylated anti-IFN-γ antibody (BD Pharmingen, clone XMG 1.2), streptavidin-conjugated enzyme (PK-6100, Vector Laboratories) and AEC substrate (SK-4200, Vector Laboratories). The spot forming units (SFU) were enumerated using ELISpot reader (Cellular Technology Ltd, Cleveland, OH).

Limiting dilution analysis

The cytotoxic potential of CD8⁺ T cells was estimated using limiting dilution analysis (LDA) (240, 241). Effector cells were derived from the lungs and lung draining lymph nodes of *M. tuberculosis*-infected mice at designated time points. Freshly isolated cells were plated in 2-fold serial dilutions starting from 40,000 cells/well to 1250 cells/well in V-bottom 96-well plates (24 replicates/input number) supplemented with IL-2 at 20 U/ml. Lung and lymph node cells were incubated with *M. tuberculosis*-infected dendritic cells (500 DC/well) for 7 days. Following incubation, 100 µl of spent media was removed from each well, and lung cells were cultured for another round of stimulation with *M. tuberculosis*-infected macrophages (1000 MΦ/well) and IL-2 (20 U/ml) to allow for expansion of CTL precursors. Flow cytometry analysis revealed that 75-95% of cells were CD8⁺ T cells after 2 weeks of stimulation.

Cytotoxicity was determined in each well by a standard ⁵¹Chromium release assay with *M. tuberculosis*-infected macrophages as targets. *M. tuberculosis*-infected macrophages were

labeled with ^{51}Cr (100 μl of ^{51}Cr per 3×10^6 macrophages) for 1 hour at 37°C , and added to lung and lymph node T cell cultures at 4000 cells/well. Following 4 hour incubation, 100 μl of supernatant was collected (Skatron SCS System; Skatron, Sterling, VA) and radioactivity was quantified using a gamma counter. Positive wells were defined as being greater than mean ± 3 SD of spontaneous target cell release. Frequency of CTLp was determined using zero-order Poisson equation ($\ln Y = -Fx + \ln A$; where x = the number of effector cells/well; Y = % negative wells; A = the y-axis intercept; F = CTLp frequency defined by the negative slope of the line). All calculations were performed using a software program fitted to the equation by χ^2 minimization analysis (a generous gift from Dr. Carolyn A. Keever-Taylor, Medical College of Wisconsin).

Statistics

Statistically significant differences in the numbers of effector T cells between the two time points were determined using 2-way ANOVA. The p-value of < 0.05 was defined as being significant.

A.3 Results

IL-15 administration did not improve the survival of CD4^{-/-} mice

CD4^{-/-} mice were injected daily with 1 µg/200 µl of human IL-15 starting at 5 days post-infection until 21 days post-infection. Our data indicate that IL-15 treatment did not have a significant effect on controlling the bacterial infection or survival of susceptible CD4^{-/-} mice after low dose infection with aerosolized *M. tuberculosis*.

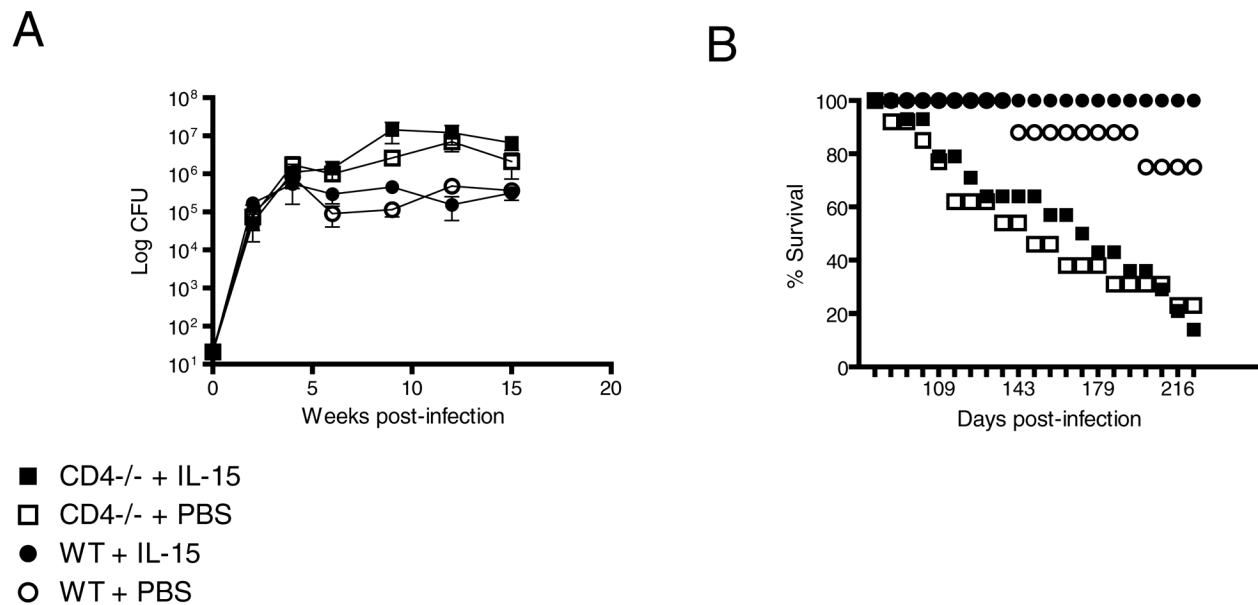


Figure 37 IL-15 administration did not affect control of bacterial replication or survival of CD4^{-/-} mice

(A) Lung homogenates from IL-15 and PBS treated CD4^{-/-} and wild-type mice were plated to determine the number of colony forming units (CFU).

(B) Survival curve.

IL-15 administration did not enhance CD8⁺ T cell effector functions in CD4^{-/-} and wild-type mice

IL-15 administration did not significantly augment the numbers of CD8⁺ T cells in either CD4^{-/-} and wild-type mice, and there is no evidence that IL-15 treatment enhanced IFN- γ production or cytotoxic activity.

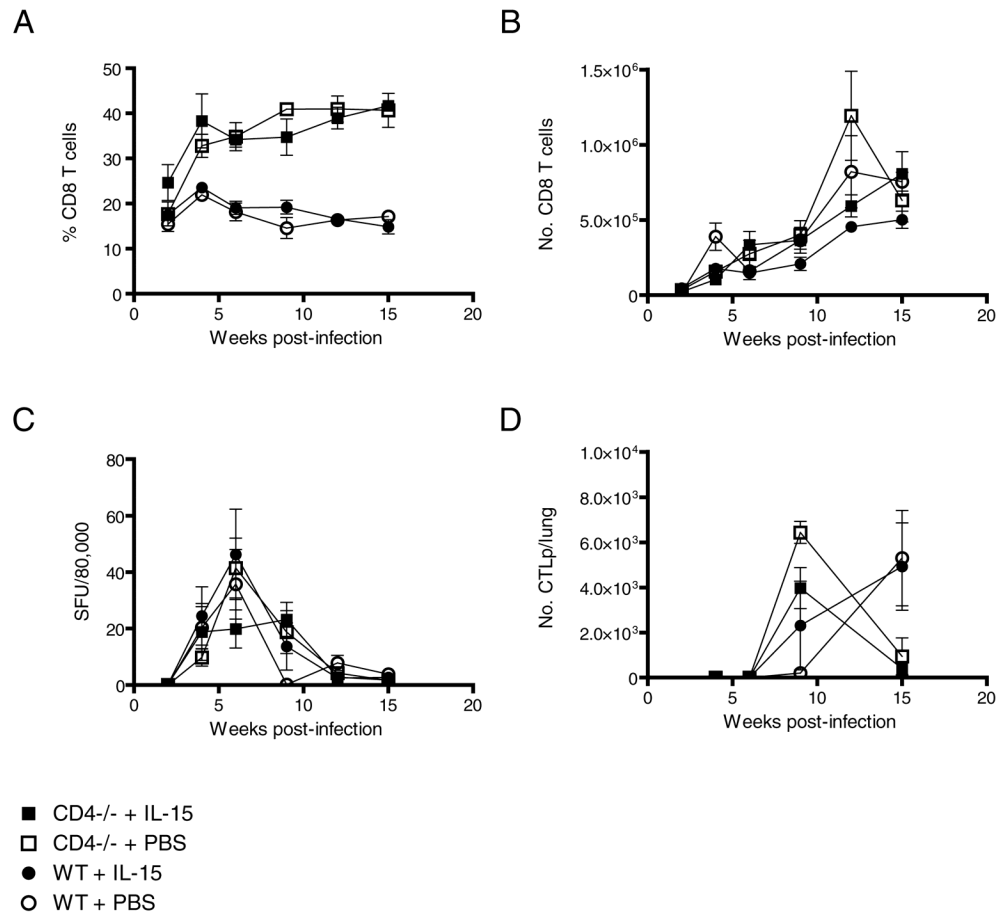


Figure 38 IL-15 administration did not enhance CD8⁺ effector functions in CD4^{-/-} and wild-type mice

(A-B) The graphs depict the percentage of CD8⁺ T cells within lymphocyte gate and the number of CD8⁺ T cells in a whole lung of IL-15 and PBS treated CD4^{-/-} and wild-type mice
 (C) Frequency of IFN- γ producing CD8⁺ T cells and (D) the number of CTLp in the lungs of IL-15 and PBS treated CD4^{-/-} and wild-type mice.

A. 4 Discussion

IL-15 treatment of susceptible CD4^{-/-} and wild-type mice did not significantly change the ability of mice to control infection or the course of the disease. IL-15 administration did not improve the survival of CD4^{-/-} mice. There was no indication that IL-15 immunotherapy increased the number of CD8⁺ T cells or enhanced CD8⁺ T cell effector functions.

It is important to note that the course of infection in this set of experiments did not follow the normal response kinetics. Until 6 weeks post-infection, the numbers of immune cells infiltrating the lungs of both CD4^{-/-} and wild-type mice were so low that not all experiments were performed with optimal numbers of cells. For example, in the setting of LDA as little as 10,000 cells/well (highest cell concentration) to 125 cells/well (lowest cell concentration) were used, most likely contributing to undetectable CTL activity during acute infection. It was not until 9 weeks post-infection that cell numbers sufficiently increased in the lungs of infected mice to set up experiments according to standard conditions. The study should be repeated just to confirm that during early time points IL-15 did not significantly influence the cytotoxic activity of CD8⁺ T cells in CD4^{-/-} and wild-type mice.

APPENDIX B

A list of submitted manuscripts and manuscripts in preparation

1. Lazarevic, V. and Flynn, J.L. (2004) Long-term control of *Mycobacterium tuberculosis* infection is mediated by dynamic immune responses. *The Journal of Immunology* (submitted).
2. Lazarevic, V. Yankura, D.J., DiVitto, S.J., and Flynn, J.L. (2004) Induction of *Mycobacterium tuberculosis*-specific primary and secondary T cell responses in IL-15^{-/-} mice. (in preparation).
3. Lazarevic, V. and Flynn, J.L. (2004) CD4⁺ T cells are necessary for sustained CD8⁺ T cell memory responses following challenge with *Mycobacterium tuberculosis*. (in preparation).

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